

**ROLE OF PUTRESCINE
IN THE REGULATION OF
ORNITHINE DECARBOXYLASE
AND CELL GROWTH**

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Title and subtitle Role of putrescine in the regulation of ornithine decarboxylase and cell growth.		
Abstract The role of putrescine (and other polyamines) in cell growth has been studied by inhibiting the activity of ornithine decarboxylase (ODC), the initial enzyme in polyamine biosynthetic pathway. Thus, Ehrlich ascites tumor cells were blocked in their polyamine synthesis by treatment with two types of ODC inhibitors, substrate analogs and aliphatic diamines. The antiproliferative effectiveness of the various ODC inhibitors used was as follows: α -difluoromethylornithine (DFMO) > putrescine > 1,3-diaminopropane (DAP) > 1,3-diamino-2-propanol (DAP-OH) > α -methylornithine (MO). The extent to which cell death participated to the antiproliferative action of substrate analogs was studied by measuring the loss of ^{125}I from mice bearing ascites tumor cells whose DNA was labeled with 5-(^{125}I)iodo-2'-deoxyuridine. These experiments showed that the antiproliferative effects of MO and DFMO were partly attributable to an increased rate of tumor cell death. Accordingly, a CFE assay showed that DFMO treatment reduced the viability of the Ehrlich ascites tumor cells grown in culture. Analysis of the antiproliferative action of MO showed that cells accumulated in the S and G2 phases of the cell cycle. Putrescine treatment was found to exert a similar cell kinetic effect, and the accumulation of cells in the S phase was found to be due to a reduction in the rate of DNA synthesis. The effects of putrescine and its lower homolog, DAP, on polyamine metabolism and cell kinetics were studied in culture. These diamines proved to be more potent inhibitors of ODC activity than DFMO. However, they were less potent in their antiproliferative action and in reducing the cellular polyamine content. Inhibition of ODC activity by putrescine was shown to be mediated by antizyme, a macromolecular endogenous inhibitor. The ODC and the antizyme activities were studied in cells separated according to their position in cell cycle. There were two peaks in ODC activity during the cell cycle; in late-G1/early-S and in late-S/G2. A decrease in ODC activity consistently occurred in mid-S phase. Maximum antizyme activity occurred in this phase of the cell cycle. These results suggest that antizyme may be involved in the regulation of ODC activity during the cell cycle.		
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Fil.kand., Mö

Akademisk avhandling som med vederbörligt tillstånd av Matematisk-Naturvetenskapliga Fakulteten vid Lunds Universitet för avläggande av filosofie doktorsexamen kommer att offentligen försvaras i Zoofysiologiska institutionens föreläsningssal, Helgonavägen 3B, Lund, onsdagen den 10 april 1985, kl 09.00.

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LUND 1985

"A naturalist's life would be a happy one if he had only to observe and never to write"

Charles Darwin

The present thesis is based on the following papers:

- I. Heby O, Anehus S, Linden M and Oredsson S.
Tumor Cell Proliferation as Affected by Changes in Intracellular and Extracellular Polyamine Levels.
In: Advances in Polyamine Research, Vol 3, Caldarera C M, Zappia V and Bachrach U (eds), Raven Press, New York, pp 357-364, 1981.
- II. Linden M, Oredsson S and Heby O.
Cytocidal Effect of α -Methylornithine, a Competitive Inhibitor of Ornithine Decarboxylase, on Ehrlich Ascites Tumor Cells In Vivo.
Cancer Lett 9, 207-212, 1980.
- III. Oredsson SM, Linden M and Heby O.
Increased Rate of Tumor Cell Death Caused by Polyamine Synthesis Inhibitors.
Virchows Arch (Cell Pathol) 47, 131-138, 1984.
- IV. Linden M, Andersson G and Heby O.
Putrescine Administration Reduces the Rate of DNA Synthesis in Ehrlich Ascites Tumor Cells In Vivo. Int J Biochem 12, 387-393, 1980.
- V. Linden M, Oredsson SM, Anehus S and Heby O.
Inhibition of Ornithine Decarboxylase Activity and Cell Growth by Diamines. A Comparison Between the Effects of Two Homologs, 1,3-Diaminopropane and 1,4-Diaminobutane (Putrescine).
Submitted for publication.
- VI. Linden M, Anehus S, Långström E, Baldetorp B and Heby O.
Cell Cycle Phase-Dependent Induction of Ornithine Decarboxylase-Antizyme.
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These papers will be referred to by their Roman numerals.

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1. ABBREVIATIONS

CFE	colony forming efficiency
DAP	1,3-diaminopropane
DAP-OH	1,3-diamino-2-propanol
DFMO	α -difluoromethylornithine
(125 I)IdUrd	5-(125 I)iodo-2'-deoxyuridine
MGBG	methylglyoxal-bis(guanylhydrazone)
MO	α -methylornithine
ODC	ornithine decarboxylase
SAMDC	S-adenosylmethionine decarboxylase

2. BACKGROUND

2.1 Polyamines

The history of the three most common polyamines began already in 1678 when Antoni van Leeuwenhoek made the first microscopic analysis of human spermatozoa, described in his famous letter to the Royal Society in London (Van Leeuwenhoek, 1678). In this letter he also reported the gradual formation of colorless crystals, which in 1888 were named "spermine" by Ladenburg and Abel (1888). In 1924 to 1927 Rosenheim and his collaborators isolated spermine from various mammalian tissues. In addition they found a related compound, which they named "spermidine" (Rosenheim, 1924; Dudley et al, 1927 a, b). Yet another related compound was isolated from decomposing animal tissue. It was named "putrescine" because of its foul smell of "putrefaction".

The structures of these three aliphatic, nonprotein, bases (the polyamines) are shown in Figure 1. At physiological pH the primary as well as the secondary amino groups are protonated, giving the polyamines 2-4 positive charges. The polyamines are found in all living organisms. Their intracellular synthesis (Fig. 2) is controlled by many diverse mechanisms. Changes in the polyamine concentrations are associated with a number of important cell activities. There is a large body of evidence implicating the polyamines in the control of cell growth, but their function at the molecular level is less well understood.

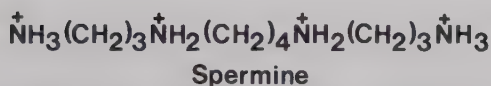
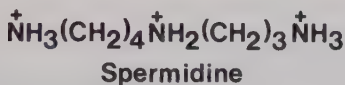
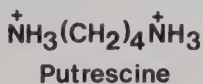


Figure 1. The molecular structures of putrescine, spermidine and spermine, the three major polyamines in mammalian cells. At physiological pH the primary and secondary amino groups are protonated.



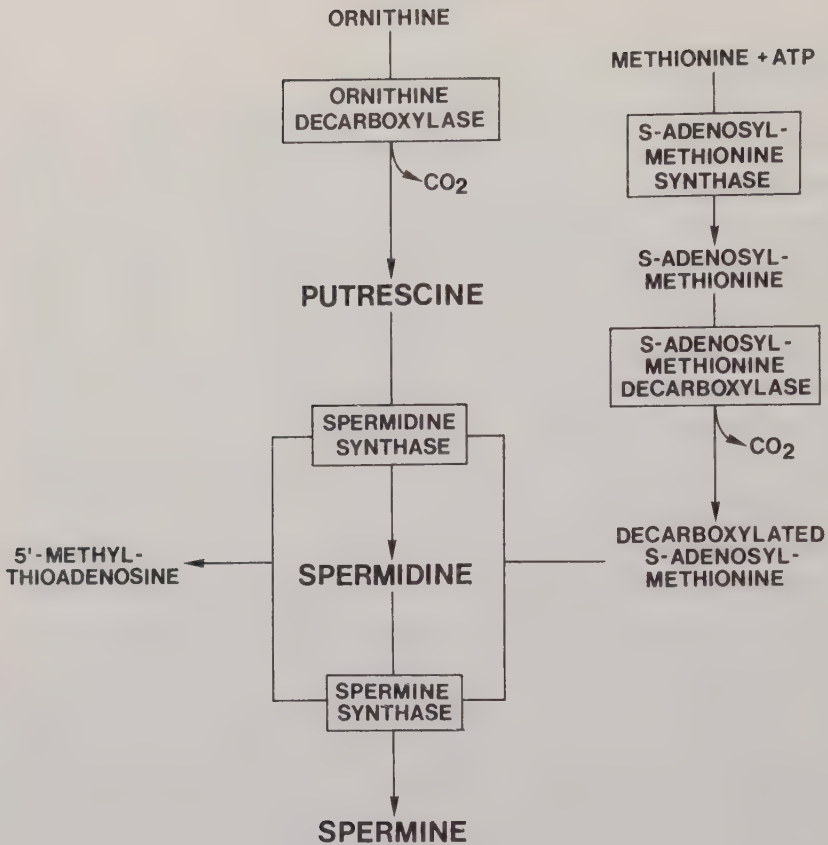


Figure 2. The polyamine biosynthetic pathway in mammalian cells.

2.2 Polyamine synthesis inhibitors

The development of inhibitors of the polyamine biosynthetic enzymes, has opened the possibility to specifically deplete cells of their polyamines (Heby and Jänne, 1981) and thus to determine the functions of the polyamines in cell growth and in other fundamental biological processes. Many inhibitors have been synthesized (for review see Heby and Jänne, 1981), but only the ones used in the present study are described.

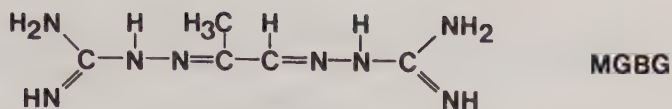


Figure 3. The molecular structure of methylglyoxal-bis(guanyldihydrazone) (MGBG), a potent inhibitor of SAMDC.

Methylglyoxal-bis(guanyldihydrazone) (MGBG, Fig. 3) was the first polyamine synthesis inhibitor to be synthesized. It is an extremely potent inhibitor of eucaryotic S-adenosylmethionine decarboxylase (SAMDC, Fig. 2). MGBG treatment blocks the formation of spermidine and spermine in the cells but increases their putrescine content. The increase in putrescine content is partly due to the fact that MGBG is a very potent inhibitor of diamine oxidase (Hölttä et al, 1973; Pegg and McGill, 1978). The effects exerted by MGBG may not be entirely due to polyamine synthesis inhibition. Thus, it has been demonstrated that some effects of MGBG (even those produced by micromolar concentrations) cannot be prevented or reversed by addition of polyamines (Porter et al, 1981).

In an attempt to find a potent and specific inhibitor of ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17, ODC, Fig. 2), the first and rate-limiting enzyme in polyamine biosynthesis, many substrate and product analogs have been synthesized. α -Methylornithine (MO, Fig. 4) proved to be a potent, competitive and reversible inhibitor of ODC (Bey et al, 1978). MO treatment reduces the cellular putrescine and spermidine content. However, MO exerts some unwanted effects; it stabilizes ODC and increases the SAMDC activity and the spermine content. α -Difluoromethylornithine (DFMO, Fig. 4) is an enzyme-activated irreversible inhibitor of ODC (Metcalf et al, 1978). It is decarboxylated by ODC, but not by other decarboxylases, and it binds covalently to the active site of the enzyme. As a consequence, DFMO is the most potent and specific inhibitor of putrescine synthesis available to date. Nevertheless, it has some unwanted effects. Thus, DFMO increases the SAMDC activity (Mamont et al, 1982). This fact in combination with the decrease in putrescine and spermidine content leads to a dramatic accumulation of decarboxylated S-adenosylmethionine (Pegg et al, 1982). Another unwanted effect of DFMO is that it increases the spermine content (Oredsson et al, 1980).

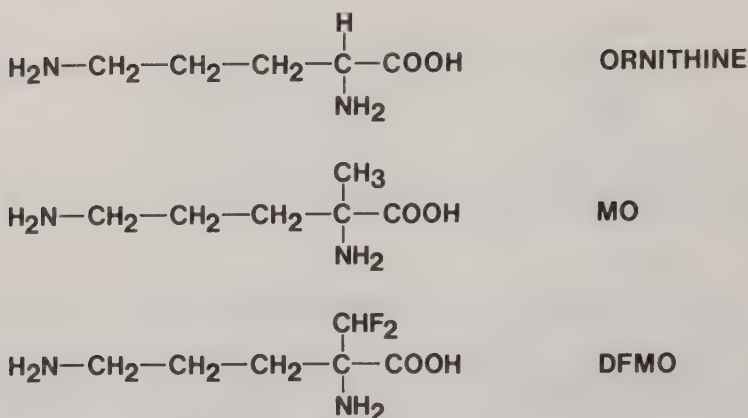


Figure 4. The molecular structures of two ornithine analogs: α -methyl-ornithine (MO), a competitive and reversible inhibitor of ODC; and α -difluoromethylornithine (DFMO), an enzyme-activated and irreversible inhibitor of ODC.

Amines represent another type of ODC inhibitors. The concept of amine inhibition of ODC activity has been developed on the basis of the observation by Pett and Ginsberg (1968), that low concentrations of exogenous putrescine strongly inhibit the ODC activity. In addition to putrescine, its lower homolog 1,3-diaminopropane (DAP, Fig. 5) and 1,3-diamino-2-propanol (DAP-OH, Fig. 5), are potent ODC inhibitors (Pösö and Jänne, 1976; Kallio, 1978; Piik et al, 1978). Putrescine does not exert its effect on ODC as a product inhibitor because the K_i is too high (1 mM) (Jänne and Williams-Ashman, 1971). Instead the amine action on ODC appears to be indirect. Amines may act through

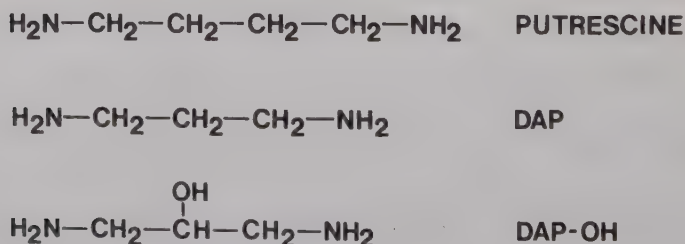


Figure 5. The molecular structures of 1,4-diaminobutane (putrescine), 1,3-diaminopropane (DAP) and 1,3-diamino-2-propanol (DAP-OH), potent inhibitors of ODC acting through an indirect mechanism.

transcriptional or translational control mechanisms (Kallio et al, 1977 b) or by inducing endogenous protein inhibitors of ODC (Fong et al, 1976).

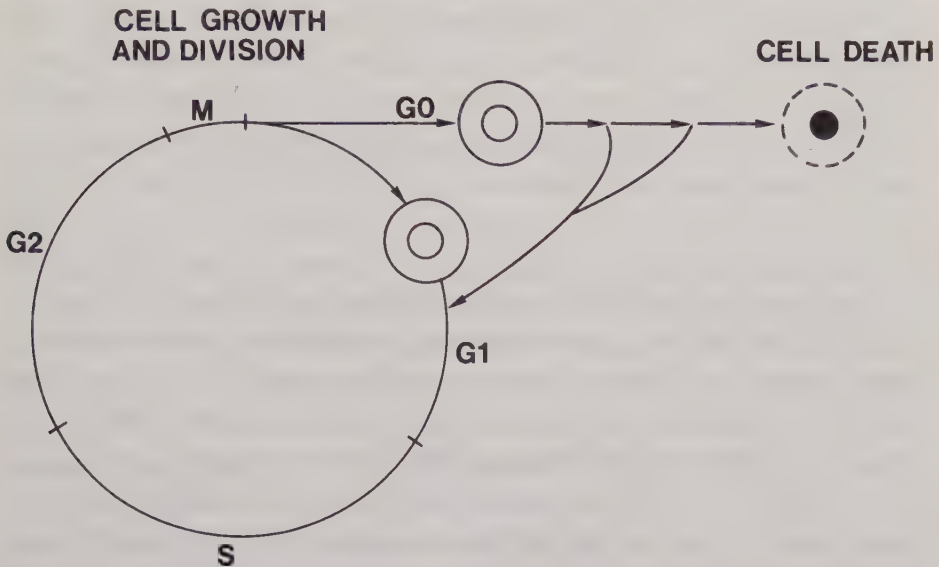


Figure 6. The mammalian cell cycle.

2.3 Polyamines in the regulation of cell growth

The life cycle of normal or tumor cells, which serves to double all the structural elements, begins after cell division. The daughter cells either enter the G1 ("gap 1") phase or leave the cell cycle by entering a G0 ("resting") phase. Cells in G0 may reenter G1, but this becomes increasingly difficult as they progress further into G0. After progression through the G1 phase the cells enter the S ("synthetic") phase, during which the amount of cellular DNA is doubled. After completion of DNA synthesis, the cells enter the G2 ("gap 2") phase of the cell cycle, in preparation for mitosis (M) (Fig. 6).

Stimulation of cell growth and division is associated with increased polyamine biosynthesis (Cohen, 1971; Bachrach, 1973; Jänne et al, 1978). Earlier studies have revealed that there are two bursts

of polyamine synthesis during the cell cycle, one during late-G1/-early-S and another during late-S/G2 (Heby et al, 1976). The involvement of polyamines in DNA synthesis and mitosis has been studied using specific inhibitors of polyamine biosynthesis. These studies revealed that polyamine depletion inhibits DNA replication and cell division (cytokinesis). Polyamine synthesis inhibition may also exert adverse effects on other major events in cell growth and division, e.g. RNA and protein synthesis (Heby and Jänne, 1981). In view of their anti-proliferative effect the polyamine synthesis inhibitors are potentially useful in cancer therapy.

2.4 Mammalian ODC

In mammalian cells putrescine is formed only in the reaction catalyzed by ODC. The agmatine pathway has not been demonstrated in animal cells. ODC catalyzes the decarboxylation of L-ornithine to putrescine, the precursor of spermidine and spermine (Cohen, 1971) (Fig. 1). Pegg and Williams-Ashman (1968) first demonstrated the presence of ODC activity in a mammalian tissue (rat prostate). This report was followed by two simultaneous papers demonstrating ODC activity in regenerating rat liver (Jänne and Raina, 1968; Russell and Snyder, 1968). Analyses of partly purified ODC showed that the enzyme is dependent on pyridoxal 5'-phosphate (Pegg and Williams-Ashman, 1981) and on low molecular weight thiols (Jänne and Williams-Ashman, 1971). In the absence of thiol compounds, ODC rapidly loses its activity as a result of polymerisation (Jänne and Williams-Ashman, 1971).

The ODC activity is extremely low in quiescent cells. However, it can be rapidly elevated by exposure to various trophic stimuli, e.g. hormones, growth factors and drugs (Jänne et al, 1978; Canellakis et al, 1979; McCann, 1980; Russell, 1980). Even when maximally induced, ODC represents only about 0.01% of the total cellular protein content (Pegg and McCann, 1982). The enzyme is induced within just a few hours after growth stimulation and has the shortest half-life (as short as 5-10 min) among enzymes (Russell and Snyder, 1969; Pegg and Williams-Ashman, 1981).

The low quantities of ODC in tissues and its extreme lability (Pegg and Williams-Ashman, 1968) have been major reasons for difficulties in purification and in studies of its regulation. Nevertheless,

ODC has been recently purified to apparent homogeneity from mouse kidney (Persson, 1981 a,b; Seely et al, 1982, Isomaa et al, 1983) and rat liver (Kameji et al, 1981; Kitani and Fujisawa, 1983). The characteristics of purified ODC are summarized in Table 1. The purified enzyme preparation exhibits a specific activity that is in agreement with the theoretical activity of pure enzyme (Persson 1981 b).

Antibodies to rat liver ODC were produced in many laboratories (Hölttä, 1975; Theoharides and Canellakis, 1976; Kallio et al, 1977 a; Obenrader and Prouty, 1977). However, these antibodies were raised using ODC preparations with lower specific activities than more recent ODC preparations (Persson, 1982; Seely et al, 1982; Kameji et al, 1981; Kitani and Fujisawa, 1983). The specificity of the antibodies was not fully established. With the use of ODC preparations from mouse kidney and rat liver monospecific antibodies have been generated (Persson, 1981 b; Seely and Pegg, 1983 a; Kameji et al, 1981; Isomaa et al, 1983). These antibodies permitted the development of a radio-immune assay (Seely and Pegg, 1983 a; Isomaa et al, 1983) which is a very useful tool in studies of ODC regulation.

The isolation of monospecific ODC antibodies and the synthesis of (5-³H)DFMO, made it possible to study the intracellular localization of ODC by two independent microscopic techniques (Anehus et al, 1984 a). However, these two methods revealed divergent ODC distribution patterns. The immunocytochemical approach (using ODC antibodies) indicated that a majority of the cells had a much higher concentration of ODC protein in the cytoplasm than in the nucleus, while the autoradiographic approach (using (5-³H)DFMO) indicated that the concentration of catalytically active ODC was about the same in nucleus and cytoplasm (Anehus et al, 1984 a).

2.5 Regulation of ODC

It has been noted in many experimental systems that the ODC activity is regulated very precisely (for review see McCann, 1980). It may be regulated at many levels: transcriptional, post-transcriptional, translational and/or post-translational ones.

The role of RNA synthesis in ODC induction has been investigated by using various RNA synthesis inhibitors. Experiments with actinomycin D are inconclusive, because the ODC activity was found to be

Table 1. Characteristics of purified mammalian ODCs.

Source	Specific activity (nmol/hr/mg prot)	Purification (-fold)	Molecular weight ($\times 10^3$)	K_m (PLP) (μ M)	K_m (Orn) (μ M)	pI	Reference
mouse kidney	1 389 000	2 266	N.D.	N.D.	N.D.	N.D.	Persson (1981)
mouse kidney	2 980 000	10 350	100 (53)*	0.3	75	4.8	Seely et al. (1982)
mouse kidney	1 818 000	2 139	N.D. (54)*	N.D.	N.D.	4.7-4.9	Isomaa et al. (1983)
rat liver	1 100 000	350 000	105 (50)*	N.D.	N.D.	4.1	Kameji et al. (1982)
rat liver	1 464 000	710 000	145 (54)*	0.09 4.7	60	5.7	Kitani et al. (1983)

* Subunit molecular weight in parenthesis.

N.D. = not determined

PLP = pyridoxal 5'-phosphate

Orn = ornithine

either inhibited, unaffected or superinduced by treatment with this DNA-dependent RNA synthesis inhibitor (for review see McCann, 1980). Cordycepin (an inhibitor of mRNA elongation and polyadenylation) and α -amanitin (an inhibitor of RNA polymerase II), however, were consistently found to reduce the ODC activity in various animal systems (Clark 1974; Heby and Emanuelsson, 1980). Therefore it appears that the ODC activity is at least partly dependent on RNA synthesis.

Recently developed techniques, using complementary DNA (cDNA) probes, now permit direct analysis of ODC mRNA concentrations (Kontula et al, 1984; Berger et al, 1984). These studies and experiments in which hepatic polysomes were used as an mRNA source (Kameji et al, 1984) revealed that induction of ODC activity in mouse kidney and in rat liver is associated with an increase in ODC mRNA concentration.

A rapid post-translational control of ODC activity by exogenous polyamines has been demonstrated in many experimental systems (for review see McCann, 1980). The experimental evidence shows that low (μ M) putrescine concentrations are sufficient to block the ODC activity in situ even though putrescine is merely a weak competitive inhibitor (Pegg and Williams-Ashman, 1968; McCann et al, 1977). ODC inhibition by polyamines is associated with disappearance of ODC immunoreactive protein (Canellakis and Theoharides, 1976; Kallio et al, 1977 a). However, the exogenous polyamines do not control the ODC activity only by inhibiting enzyme synthesis. They also may induce an ODC inhibitory protein, termed "antizyme". Antizyme, which inactivates ODC by complex formation (Heller et al, 1976), was first described by Fong et al (1976). Antizyme regulation of ODC activity is described in greater detail in the next paragraph (2.6). Further studies in this field, recently showed that antizyme-inactivated ODC can be reactivated by a macromolecular inhibitor of the antizyme (Fujita et al, 1982).

Another mechanism for post-translational modification of ODC activity is the transglutaminase-mediated incorporation of putrescine into ODC (Folk, 1980; Russell, 1981; Delcros et al, 1984). Yet another, is the protein kinase-catalyzed phosphorylation of ODC (Atmar and Kuehn, 1981). Phosphorylation of the enzyme has been observed also in experiments where *E. coli* ODC was treated with polyamine-dependent protein kinase from pig epidermal cells (Nemoto et al,

1984). These findings suggest that the turn-over of ODC might be regulated by phosphorylation of the enzyme.

Other means of post-translational control of ODC have been suggested by Clark and Fuller (1976 b). They reported the existence of two forms of ODC with respect to pyridoxal 5'-phosphate affinity.

The fact that there is a multitude of intricate mechanisms of ODC regulation in the cell emphasizes the importance of this enzyme in polyamine biosynthesis and in cell growth. Moreover, it has been proposed that ODC is a multifunctional protein that may have a specific regulatory role in rRNA gene transcription in the nucleolus (Atmar and Kuehn, 1981). Thus, the phosphorylation of ODC might have a general effect on ribosomal RNA synthesis in addition to the prevention of polyamine biosynthesis.

2.6 Mammalian antizyme

The induction of ODC inhibitory protein by addition of putrescine to cell cultures was discovered by Fong et al (1976). This protein was termed "antizyme" because it was released and/or synthesized in response to proximal or distal products of the ODC-catalyzed reaction (Heller et al, 1976).

Antizyme was shown to be a noncompetitive inhibitor of ODC with an approximate molecular weight of 26,500. It inhibited ODC by forming a complex, which was stable during Sephadex chromatography. When treated with high salt concentrations, however, the enzyme and the inhibitor dissociated. Antizyme is heat-labile and trypsin-sensitive, and its induction is inhibited by cycloheximide but not by actinomycin D. It has as short a half-life as ODC (derived from the same source) and it crossreacts with ODC from other cells (Heller et al, 1976).

Antizyme has been partially purified (1,600-fold) from rat liver (Canellakis et al, 1981) and very recently to apparent homogeneity (600,000-fold) from the same source (Kitani and Fujisawa, 1984). Homogeneous antizyme is very labile, but is stabilized by Tween 80 and mercaptoethanol. Its molecular weight is 19,000-22,000. It is a single polypeptide chain with an isoelectric point of 6.8, which differs from those previously reported (pI 5.5 - 6.5) (Canellakis et al, 1978). The equilibrium constant of the reaction between antizyme and ODC is as high as $1.4 \times 10^{11} \text{ M}^{-1}$. Antizyme has a broad pH optimum (7-8)

and its reaction with ODC is slower at 37°C than at 0°C.

Recently, it has been reported that a macromolecular inhibitor of antizyme is present in rat liver. This inhibitor not only inhibits antizyme but also reactivates ODC (Fujita et al, 1982). The antizyme inhibitor may be used for quantitative analysis of antizyme (Fujita et al, 1984; Brosnan et al, 1983).

The induction of antizyme by exogenous di- and polyamines and their synthetic analogs has been observed in various experimental systems (McCann et al, 1977; Branca and Herbst, 1980; Heller and Canellakis, 1981). However, antizyme induction was not detected in 3T3 cells (Clark and Fuller, 1976 a), in rat kidney, prostate and heart (Pegg et al, 1978) or in mouse kidney (Persson and Rosengren, 1979). The concentration of the antizyme inducer (e.g. putrescine) required to completely inhibit the activity of ODC is characteristic of each cell type and may vary by as much as 1,000-fold. Antizyme can be detected in cells treated with as little as 0.1 μ M putrescine (Heller et al, 1978). Antizyme was demonstrated to be a normal component of H-35 cells and rat liver (that had not been treated with putrescine or other diamines) when homogenates were extracted with polyamines (Heller et al, 1977). Moreover, antizyme was detected without treatment with exogenous polyamines, in mouse brain (Hietala, 1983), rat skin (Lesiewicz and Goldsmith, 1983), rat mammary gland (Brosnan et al, 1983) and rat liver (Fujita et al, 1984).

These results indicate that antizyme is involved in the regulation of ODC in the cell. It has been postulated that antizyme might make ODC available to a specific fast-acting proteolytic system (Heller et al, 1976; Seely and Pegg, 1983 b). Another interesting hypothesis is that proposed by Atmar and Kuehn (1981), namely, that antizyme is identical with the catalytic subunit of a polyamine-dependent protein kinase that phosphorylates ODC.

3. THE PRESENT STUDY

3.1 SPECIFIC AIMS

The physiological role of the polyamines may be analyzed by evaluating the consequences of depletion and subsequent replenishment of the cellular polyamine content.

In the present study, ornithine analogs and aliphatic diamines were used to inhibit the activity of ODC, the initial enzyme in the polyamine biosynthetic pathway. The effects of these ODC inhibitors on cell growth (I) and viability (II,III) were evaluated using Ehrlich ascites tumor cells grown in vivo and in culture.

Because putrescine and DAP inhibited the ODC activity more efficiently than did MO and DFMO, the effects of these diamines were analyzed in greater detail. These studies included analyses of polyamine metabolism and cell kinetics (IV, V).

Finally, experiments were designed to answer the important questions whether the ODC inhibitory effect of putrescine is antizyme-mediated (IV) and whether antizyme is involved in the regulation of ODC activity during the cell cycle (VI).

3.2 MATERIALS AND METHODS

3.2.1 Ehrlich ascites tumor cells grown in vivo (II, III, IV, VI)

The Ehrlich ascites tumor was originally derived from a solid transplantable mouse mammary carcinoma (Ehrlich and Apolant, 1905). For the present studies, a hyperdiploid subline, Ehrlich-Lettre-Diploid Copenhagen, was used. This strain of the tumor was obtained from the Department of Genetics at the University of Lund (Andersson and Agrell, 1972). The ascites tumor has been maintained in males of an inbred albino mouse strain (NMRI) by i.p. transplantation every 7-12 days into new (6-week-old) hosts. Each mouse received 4×10^6 cells suspended in 0.4 ml of 0.9% NaCl.

3.2.2 Ehrlich ascites tumor cells grown in culture (I, III, V)

Tumor cells were collected aseptically from the peritoneal cavity of a tumor-bearing mouse at 6 days after tumor transplantation. The cells were adapted to growth in suspension culture.

The growth medium (Swim's S-77), contained 10% fetal calf serum and various supplements which are described in paper III. The cell cultures were incubated without agitation at 37°C in a water-saturated atmosphere of 95% air and 5% CO₂. The tumor cells were grown under these conditions for about 30 passages without any apparent changes in their growth characteristics. The cells were routinely subcultured every 3 or 4 days by a 20-fold dilution with fresh medium to 1×10^5 cells/ml (I, III, V).

3.2.3 Collection and counting of cells (I-VI)

Tumor cells grown in mice were collected by rinsing the peritoneal cavity with 10 ml of ice-cold phosphate-buffered saline (PBS) (II, III, IV, VI). Cells grown in culture were counted after removal of an aliquot of the cell suspension (I, V). Cells were counted in a Coulter counter (I, V) or in a Bürker chamber (II, III, IV, VI). Cells were harvested by centrifugation and the cell pellets were stored at -20°C, except those intended for ODC, antizyme and SAMDC analyses, which were stored at -70°C.

3.2.4 Cell size distribution analysis (I, V)

Approximately 1×10^6 cells were suspended in 100 ml of 0.9% NaCl containing 1% formaldehyde. The cell distribution was analyzed electronically using a Coulter counter (Industrial Model D).

3.2.5 Centrifugal elutriation (VI)

Centrifugal elutriation, or counterflow centrifugation, was first developed by Lindahl (1948). More recently, Beckman Instruments have constructed an elutriator rotor (Model JE-6), which is commercially available. Centrifugal elutriation may be used either to separate various types of cells in a population (Flangas, 1974; Glick et al, 1971; Grabske et al, 1975) or to separate cells according to their position in the cell cycle (Keng et al, 1980; Bjerkvig et al, 1983).

A heterogenous cell population is suspended in a separation chamber by balancing of an outwardly directed centrifugal force and an inwardly directed force produced by the continuous flow of fluid. Suspended cells reach their equilibrium position in the separation chamber according to the sedimentation velocity, which is based on the size, density and shape of the cells. When either the rotor speed is decreased or the velocity of the fluid flow is increased, the balance of forces shifts and so does the equilibrium position of all cells. Those cells with lowest sedimentation rate flow out of the chamber and are collected. Densities and shapes of cultured mammalian cells are reasonably constant throughout the cell cycle (Andersson et al, 1970), but differences in cell size (Steen and Lindmo, 1978) permit the separation (by centrifugal elutriation) of the cells according to their position in the cell cycle.

The basic elements of the elutriator system used are shown in Figure 7. Flow rates were calibrated by measuring the fluid volume collected per unit time at specific dial settings. Ehrlich ascites tumor cells were separated by centrifugal elutriation according to a modification (Anehus et al, 1983) of the method developed by Grabske et al (1975), as described in paper IV.

3.2.6 Tumor cell death (II, III)

The rate of tumor cell death was studied in vivo by following the loss of radioactivity from mice bearing tumor cells labeled

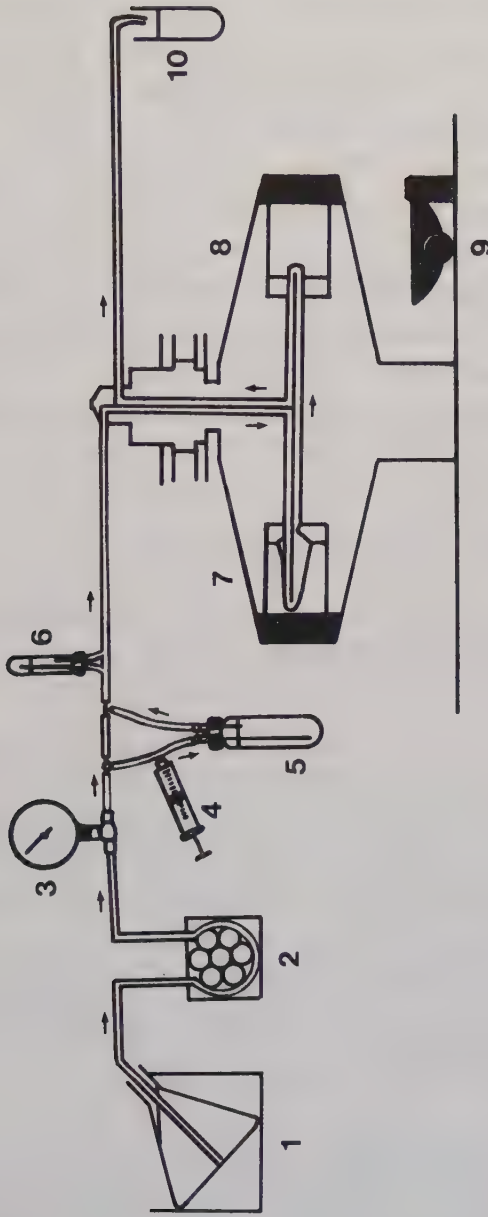


Figure 7. Centrifugal elutriation system for separation of cells according to their size. (1) Reservoir with ice-cold medium; (2) peristaltic pump; (3) pressure gauge; (4) syringe for cell sample injection; (5) sample mixing chamber; (6) bubble trap; (7) separation chamber and (8) bypass chamber of the Beckman JE-6 elutriator rotor; (9) stroboscope; (10) fraction collector.

exclusively in their DNA with 5-(^{125}I)iodo-2'-deoxyuridine ((^{125}I)IdUrd) (Hofer et al, 1969 a,b; Commeford and Joel, 1979) (II, III).

The cellular DNA of 3-day-old tumors was labeled by 2 consecutive i.p. injections of 185 kBq of (^{125}I)IdUrd given 20 hr apart (II, III). Labeling of the DNA was improved by simultaneous administration of 5'-fluorouracil (III).

The accumulation of ^{125}I in the thyroid of the mice was prevented by supplementing their drinking water with 0.1% NaI, beginning 2 days before the transplantation of labeled tumor cells. Immediately after tumor cell transplantation and at daily intervals thereafter the whole body ^{125}I -radioactivity was monitored by γ -counting of the individual live mice in a well-type scintillation counter. The rate of ^{125}I -excretion was taken as an index of tumor cell death (Hofer et al, 1969 a,b) (II, III).

3.2.7 Colony forming efficiency (CFE) assay (III)

To analyze the viability or clonogenic capacity of Ehrlich ascites tumor cells grown in culture a CFE assay in soft agar was used. A cell is considered clonogenic if it can proliferate to form a colony.

The CFE of Ehrlich ascites tumor cells was determined for untreated cells and for cells treated with DFMO for 48 hr. Thus, petri dishes were covered with a layer of 0.5% agar in complete medium. On top of this layer 0.3% agar in complete medium, with a known number of untreated or DFMO-treated cells, was added. After 14 days of incubation (37°C; 5% CO_2 in air) the colonies were counted using a stereo microscope. A colony was defined as a cluster containing 50 cells or more. The CFE was the number of colonies counted divided by the number of cells seeded.

3.2.8 Flow cytometric analysis (V, VI)

Cells, harvested by centrifugation (500 x g; 5 min) were fixed in absolute ethanol ($1-2 \times 10^6/\text{ml}$) at -20°C and analyzed for their cell cycle distribution by flow cytometry. Prior to analysis, the cells were treated with pepsin, RNase and Nonidet P40 and stained with propidium iodide as previously described (Anehus et al, 1984 b).

Propidium iodide selectively and quantitatively intercalates into the double-stranded regions of nucleic acids. In RNase-treated cells its fluorescence intensity is proportional to the DNA content. The stained cell nuclei were analyzed using a Cytofluorograph 50-H (Ortho Instruments) equipped with a 4 W argon-ion laser (Model 95). About 2×10^4 cells from each sample were analyzed.

The percentages of cells in each cell cycle phase were derived from the DNA histograms essentially as described by Dean (1980).

3.2.9 DNA synthesis analysis (IV)

DNA synthesis was studied by determining of the rate of (^3H)thymidine incorporation into acid-insoluble material (IV).

The labeling index, i.e. the percentage of labeled cells, was estimated by autoradiography as described by Andersson (1977).

Photometric measurements on autoradiographs were used to estimate the relative amount of (^3H)thymidine incorporated per cell according to the method described by Fujita et al (1974).

3.2.10 Mitotic index (IV)

The mitotic index, i.e. the percentage of cells in mitosis, was determined by counting at least 500 cells in each sample.

3.2.11 ODC activity assay (IV, V, VI)

The ODC activity was measured by trapping the $^{14}\text{CO}_2$ released enzymatically from DL-(1- ^{14}C)-ornithine in the presence of saturating levels (1 mM) of L-ornithine (Jänne and Williams-Ashman, 1971).

For this assay the cells were disrupted by sonication in homogenization medium (pH 7.2) containing 10 mM Tris-HCl, 5 mM dithiothreitol (to maintain the sulfhydryl groups and thus prevent the polymerization of the enzyme), 0.5 mM Na_2EDTA and 0.05 mM pyridoxal 5'-phosphate.

The reaction mixture contained 10 mM Tris-HCl (pH 7.2), 5.5 mM dithiothreitol, 0.2 mM pyridoxal 5'-phosphate, 0.05 mM Na_2EDTA , 1 mM L-ornithine, 0.5 μCi of DL-(1- ^{14}C)ornithine monohydrochloride (specific activity 59 mCi/mmole) and 100 μl of cell extract in a total volume of 1.00 ml. The reaction was carried out in test tubes equipped

with rubber stoppers. The $^{14}\text{CO}_2$ released during 60 min of incubation at 37°C was trapped in 0.1 ml of 1 M Hyamine hydroxide contained in a polypropylene well attached to the rubber stopper. By the injection of 0.5 ml of 40% trichloroacetic acid through the rubber stopper the reaction was terminated, and bound $^{14}\text{CO}_2$ was released from the reaction mixture. A further incubation for 30 min at 37°C was allowed to ensure that all $^{14}\text{CO}_2$ released during the enzymic reaction was trapped in the Hyamine hydroxide. The polypropylene wells and their contents were then removed and placed in counting vials containing 10 ml of liquid scintillation fluid. Radioactivity was assayed using a liquid-scintillation spectrometer. The counting efficiency was in the range of 90-95%. Corrections were applied for radioactivity of non enzymic control samples (100 μl of homogenization medium replacing the tumor cell extract). In calculating the amount of ornithine decarboxylated the D isomer was assumed not to be decarboxylated by ODC (Pegg and Williams-Ashman, 1968).

3.2.12 Antizyme activity assay (IV, VI)

The antizyme activity was determined mainly as described by Fong et al (1976). The assay is basically the same as the ODC assay with some exceptions. Thus, the cells were disrupted in homogenization medium supplemented with 250 mM NaCl. A 100 μl aliquot of an enzyme preparation (45,000 $\times$ g supernatant), obtained from growth-stimulated cells with known ODC activity, was included in the reaction mixture. The extent of inhibition exerted by the antizyme preparation, in percent of added ODC activity, was determined (IV). Only free antizyme activity was assayed for (VI). The cell extracts were not dialyzed since this procedure did not reduce the ODC inhibitory activity. One antizyme unit is defined as the amount which inhibits one unit of ODC activity i.e. 1 nmole of $^{14}\text{CO}_2/\text{hr}$ (VI).

3.2.13 Separation of ODC and antizyme (IV)

ODC and antizyme were separated essentially according to McCann et al (1977). About 2×10^7 tumor cells were sonicated in ice-cold homogenization medium and the 45,000 $\times$ g supernatant of each cell extract was collected. Supernatants were extensively dialyzed and 1.5 ml aliquots were applied on a Sephadex G-75 Superfine column

(1.6 x 22 cm) that had been previously equilibrated with elution medium (5mM dithiothreitol, 0.5 mM Na₂EDTA, 0.05 mM pyridoxal 5'-phosphate and 250 mM NaCl in 10 mM Tris-HCl buffer, pH 7.2). After collection of 16 ml of eluate (the void volume) 40 fractions (325 μ l each) were collected. A 100 μ l aliquot was used for the assay of ODC or antizyme.

3.2.14 SAMDC activity assay (IV)

The SAMDC activity was determined according to the method described by Pegg and Williams-Ashman (1969). The enzyme assay, as applied in our laboratory, has been described in detail by Heby et al (1975).

3.2.15 Polyamine analysis (IV, V)

Cells were disrupted by sonication in ice-cold 0.2 M perchloric acid (PCA) (V) or in ice-cold 10 mM Tris-HCl buffer containing 5 mM dithiothreitol, 0.5 mM Na₂EDTA and 0.05 mM pyridoxal 5'-phosphate (IV). An aliquot of the latter was mixed with an equal volume of 0.4 M PCA. After 1 hr on ice, the homogenate was centrifuged at 1,000 x g for 10 min at 4°C. A 200 μ l aliquot of the supernatant was dansylated and analyzed by thin-layer chromatography (Seiler, 1970, Heby et al, 1973).

3.3 RESULTS AND DISCUSSION

By blocking the activity of ODC it is possible, at least partly, to deplete cells of their polyamines (for review see Heby and Jänne, 1981). Effects of polyamine limitation and those of subsequent polyamine supplementation were investigated in an attempt to evaluate the role of polyamines in cell growth. The antiproliferative effects of two types of ODC inhibitors, substrate analogs and aliphatic diamines, were studied using suspension cultures of Ehrlich ascites tumor cells (I). Tumor cell proliferation was largely unperturbed by treatment with 5 mM MO, a reversible inhibitor of ODC. Treatment with 5 mM DFMO, an irreversible and enzyme-activated inhibitor of ODC activity, however, resulted in a marked antiproliferative effect (I). MO-treatment

suppressed the formation of putrescine (and spermidine) only for a brief time period, while DFMO-treatment caused a rapid and permanent depletion of intracellular putrescine and spermidine. Both inhibitors caused an increase in the cellular spermine content (Oredsson et al, 1980). These results indicate that the polyamines, at least putrescine and spermidine, are essential for optimal cell growth.

Addition of 1 mM putrescine to the growth medium, after 72 hr of polyamine limitation, completely reversed the antiproliferative effect of DFMO. At variance, addition of 1 mM $MgCl_2$ did not reverse the effect of DFMO (I). Thus, the reversal by putrescine was not merely a cationic effect. DAP, a lower homolog of putrescine, added at a 5 mM concentration, was also unable to reverse the antiproliferative effect of DFMO (I). Taken together these experiments demonstrate that the antiproliferative effects are indeed due to putrescine (and spermidine) deficiency.

Treatment with 10 mM putrescine or 5 mM DAP exerted similar antiproliferative effects, which were more pronounced than that exerted by 5 mM DAP-OH (I). All three diamines, however, were considerably less potent than DFMO (I). These results are consistent with the fact that the extent of polyamine depletion produced was greater in DAP-treated cells than in DAP-OH-treated cells (unpublished). It was even greater in cells treated with DFMO (Oredsson et al, 1980).

The antiproliferative effect of putrescine, is not necessarily due to polyamine depletion, because the product of the reaction that is blocked, is provided exogenously. Putrescine-treated cells were shown to contain an excess of putrescine, even after a wash (IV,V). However, putrescine taken up by the cells may not enter the endogenous pool and thus may not participate in spermidine and spermine synthesis. In fact, putrescine treatment was demonstrated to initially decrease the cellular content of spermidine and spermine (IV, V). The effect of putrescine was not due to the formation of toxic oxidation products, inasmuch as treatment with 0.1 mM aminoguanidine, a potent inhibitor of diamine oxidase, did not reduce the antiproliferative effect of putrescine.

The nonphysiological diamines do not inhibit the ODC activity specifically. They affect the polyamine biosynthetic pathway at several steps (Heby and Jänne, 1981). When considering this fact, it seems

surprising that their effects on proliferation are only moderate ones. However, these nonphysiological diamines may, at the same time, because of their structural resemblance to the naturally occurring polyamines, partly replace these polyamines in their function.

The extent to which cell death participates to the antiproliferative action of ODC inhibitors was studied by measuring the loss of ^{125}I from mice bearing Ehrlich ascites tumor cells whose DNA was labeled with (^{125}I)IdUrd (II, III). MGBG, a competitive (with respect to SAM) inhibitor of SAMDC and a potent antiproliferative agent, was included in this study because it exhibits considerable cytotoxicity even at low doses (Dave et al, 1978). Tumor-bearing mice were treated with repeated i.p. injections (0.2 ml/mouse) of MO (2.5 $\mu\text{moles/g}$ body weight), DFMO (0.85 $\mu\text{moles/g}$ body weight), putrescine (25 $\mu\text{moles/g}$ body weight) or MGBG (0.045 $\mu\text{moles/g}$ body weight). The first drug injection was given immediately after tumor transplantation and was then repeated every 6 hr throughout the experiment. Putrescine was lethal to tumor-bearing mice when continued administered for more than 1-2 days (unpublished).

The experiments demonstrated that not only MGBG (III) but also MO (II) and DFMO (III) exerted a certain degree of cytotoxicity on Ehrlich ascites tumor cells grown in mice. Thus, the results obtained using MO and DFMO at optimal therapeutic doses, suggest that these ODC inhibitors not only slow the growth rate but also cause an increase in tumor cell death. The results should be considered in view of the fact that tumor cells growing in vivo are subjected to various host-mediated responses (Cooper et al, 1975). These could possibly contribute to a rapid elimination of tumor cells affected by the drugs.

Treatment of Ehrlich ascites tumor cells grown in culture with 5 mM DFMO for 48hr reduced the growth rate of these cells and decreased the cell viability in terms of their clonogenic capacity (III). Similar effects of DFMO-treatment on cells grown in culture as spheroids were demonstrated by Luk et al (1981) and Sano et al (1984). Thus, polyamine limitation by inhibition of ODC activity, directly or indirectly reduces the viability of tumor cells. In fact, prolonged polyamine starvation of polyamine-auxotrophic CHO cells results in irreversible growth inhibition followed by cell death (Pohjanpelto et al, 1981). The reduced viability of polyamine-depleted cells suggests

that a certain polyamine level is necessary for cell survival.

ODC inhibition by MO was found to cause an accumulation of Ehrlich ascites tumor cells in the S and G2 phases of the cell cycle (Heby et al, 1978) (II). Therefore, it was important to determine whether the inhibition of ODC activity by putrescine administration (Andersson and Heby, 1977) would have the same effect on cell proliferation. In fact, putrescine-treatment (i.p. injections of 25 μ moles/mouse/hr) of tumor-bearing mice resulted in a decreased rate of DNA synthesis and an accumulation of cells in the S phase of the cell cycle (IV).

The dose of putrescine used was chosen such that it almost completely blocked the ODC activity. The effect of putrescine on ODC activity was shown to be selective in that e.g. the activity of SAMDC was not inhibited by the same treatment (IV). This suggests that there was no general inhibition of protein synthesis. Moreover, a dose of 25 μ moles/mouse/hr only increased the cellular putrescine content by 92% (IV) which corresponds to an endogenous concentration of 1 mM at most (Andersson and Heby, 1972). When 1 mM putrescine was added directly to the *in vitro* ODC assay, only 16% of the enzyme activity was inhibited (IV). Thus, the suppression of ODC activity in putrescine-treated tumor cells could not be explained by direct product inhibition. Instead the inhibitory effect was attributed to a macromolecular inhibitor of ODC (antizyme).

Antizyme was separated by means of Sephadex G-75 Superfine chromatography in the presence of 250 mM NaCl (IV) as described by McCann et al (1977). The pattern of separation was in accordance with those obtained for other mammalian cell types in other laboratories. Conceivably, the observed deceleration in tumor cell growth rate following putrescine treatment may be due to inhibition of ODC activity by antizyme. However, the product of the reaction that is inhibited, is provided exogenously. On the other hand, the observation that the spermidine and spermine content decreased after 4 hr of putrescine treatment (IV) indicates that putrescine taken up by the cells might not participate in the synthesis of these polyamines. It might be separated from the normal endogenous putrescine pool. Probably, the decrease in cellular spermidine and spermine content contributes to the antiproliferative action of putrescine.

During growth deceleration of Ehrlich ascites tumor cells there is a release of putrescine (and other polyamines) into the ascites fluid (Heby and Andersson, 1978). In parallel there is a continuous decrease of ODC activity and a progressive lengthening of the cell cycle, mainly affecting the S phase (Andersson and Heby, 1977). These events are compatible with those observed following experimental elevation of the extracellular putrescine concentration in the ascites fluid (IV). Thus, extracellular putrescine (and probably other polyamines) may act as negative effectors in the regulation of growth in the Ehrlich ascites tumor.

The antiproliferative effects of extracellular putrescine and its lower homolog, DAP, were further investigated in Ehrlich ascites tumor cells grown in culture (V). The concentrations of putrescine (10 mM) and DAP (5 mM) were chosen such that they almost completely eradicated the ODC activity, yet did not produce any apparent cytotoxicity.

Both diamines exerted a similar inhibitory effect (20%) on tumor cell growth rate. Putrescine treatment (96 hr) caused a slight accumulation of cells in the G2 phase of the cell cycle while DAP treatment (96 hr) did not change the cell cycle distribution. Both diamines, however, caused a similar increase in cell size (V). Thus, it appears that all cell cycle phases were prolonged and that cell enlargement was due to unbalanced growth. These results are at variance with those obtained by putrescine treatment of Ehrlich ascites tumor cells grown in vivo (IV) and by DAP treatment of CHO cells in vitro (Sunkara et al, 1977), i.e. where the cells were shown to accumulate in the S phase. This discrepancy may be due to the fact that the diamines in the latter studies were given at higher, possibly toxic, doses.

Putrescine treatment caused a rapid intracellular accumulation of this diamine. Nevertheless, the cellular contents of spermidine and spermine were decreased initially (V). These results suggest that extracellular putrescine taken up by cells cannot be efficiently used in the synthesis of polyamines, at least not initially. The decrease in spermidine content may be due to the fact that putrescine, like DAP, inhibits SAMDC at high concentrations (Piik et al, 1977). The decrease in spermine content may be a result of the fact that putre-

scine acts as a competitive inhibitor of spermine synthase (Hannonen et al, 1972).

That DAP treatment depleted the cellular polyamine content more effectively than did putrescine treatment (V), and yet did not exert a more pronounced antiproliferative effect suggests that DAP may partly replace putrescine in its cellular function (Mamont et al, 1978).

Di- and polyamines, particularly putrescine, inhibit the ODC activity and simultaneously induce the release and/or synthesis of antizyme (Heller et al, 1976; McCann et al, 1977; Pegg et al, 1978). The ODC activity of Ehrlich ascites tumor cells was almost completely eradicated by 4 hr of putrescine treatment. This was true both when the cells were grown in vivo and in culture (IV, V). ODC and antizyme were isolated from cell extracts, in the absence or presence of 250 mM NaCl, using a gel filtration technique (IV). The pattern of separation was largely the same whether the extract was obtained from tumor cells grown in vivo or in culture. (Fig. 8).

The role of antizyme in the regulation of ODC activity during the cell cycle was studied in paper VI. Tumor cells, obtained from putrescine-treated and control mice at 4 hr after transplantation, were separated into fractions representing all phases of the cell cycle using a centrifugal elutriation technique.

Cell cycle-related changes in ODC (in tumor cells from control mice) and antizyme (in tumor cells from putrescine-treated mice) activities were studied in corresponding cell fractions, i.e. fractions that were almost identical in terms of their cellular DNA distributions (VI).

Maximum ODC activity was observed in fractions in which the majority of cells were in late-G1/early-S or in late-S/G2. A decrease in ODC activity was consistently observed in fractions where most cells were in mid-S phase (VI). Similar cell cycle related changes have been observed in other cell types (Heby and Jänne, 1981).

Maximum antizyme activity was observed in fractions in which the majority of cells were in mid-S phase, i.e. when the ODC activity was markedly reduced (VI).

These results suggest that antizyme may regulate the ODC activity during the cell cycle. The finding that antizyme is present in tissues that have not been treated with putrescine (Brosnan et al,

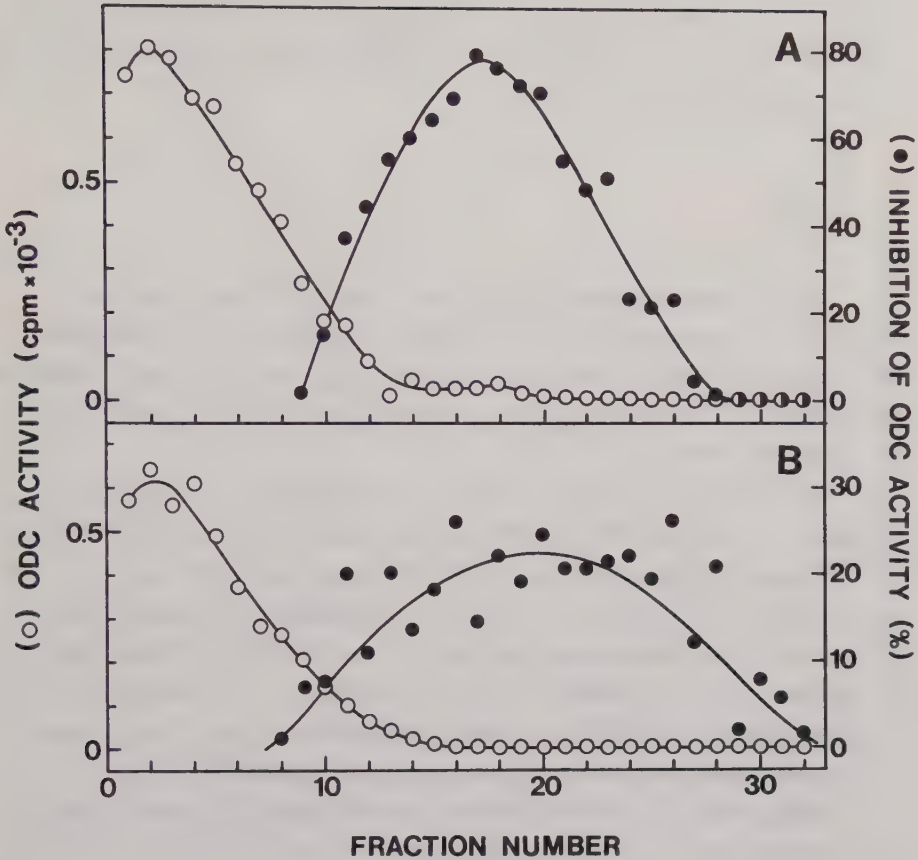


Figure 8. Comparison between antizyme induced in Ehrlich ascites tumor cells grown *in vivo* (A) and in culture (B). (A) Tumor cells were grown in the peritoneal cavity of mice. ODC activity was stimulated by transplantation of 12-day-old tumor cells into new hosts. Antizyme activity was induced by repeated injections of putrescine (25 μ moles/mouse/hr) during the first 4 hr after transplantation (IV). (B) Tumor cells were grown in culture. ODC activity was stimulated by dilution of 4-day-old cultures in fresh growth medium containing 10% fetal calf serum. Antizyme activity was induced by putrescine (10 mM) treatment during the first 4 hr after reseeding. For separation of antizyme (or ODC) a 2-ml aliquot of concentrated, dialyzed 45,000 $\times$ g supernatant (the equivalent of 3×10^8 cells) was layered onto a Sephadex G-75 Superfine column and chromatographed in the presence or absence of 250 mM NaCl.

1983; Lesiewicz and Goldsmith, 1983; Fujita et al, 1984) shows that antizyme is not an experimental artifact but is indeed involved in the normal regulation of cellular ODC activity. Antizyme-mediated modulation of the ODC activity, in which the product (putrescine) acts as the effector, may be an important regulatory mechanism involved in the control of cell growth and division.

3.4 SUMMARY

Ehrlich ascites tumor cells were blocked in their polyamine synthesis by treatment with two types of ODC inhibitors, substrate analogs and aliphatic diamines. The antiproliferative effectiveness of the various ODC inhibitors used was as follows: DFMO > putrescine > DAP > DAP-OH > MO (I).

The extent to which cell death participated to the antiproliferative action of ODC inhibitors was studied by measuring the loss of ^{125}I from mice bearing ascites tumor cells whose DNA was labeled with (^{125}I)IdUrd (II, III). These experiments showed that the antiproliferative effects of MO (II) and DFMO (III) were partly attributable to an increased rate of tumor cell death. Accordingly, a CFE assay showed that DFMO treatment reduced the viability of the Ehrlich ascites tumor cells grown in culture (III).

Analysis of the antiproliferative action of MO showed that cells accumulated in the S and G₂ phases of the cell cycle (II). Putrescine treatment was found to exert a similar cell kinetic effect, and the accumulation of cells in the S phase was found to be due to a reduction in the rate of DNA synthesis (IV).

The effects of putrescine and its lower homolog, DAP, on polyamine metabolism and cell kinetics were studied in culture. These diamines proved to be more potent inhibitors of ODC activity than DFMO. However, they were less potent in their antiproliferative action and in reducing the cellular polyamine content (V).

Inhibition of ODC activity by putrescine was shown to be mediated by antizyme, a macromolecular endogenous inhibitor (IV). The ODC and the antizyme activities were studied in cells separated according to their position in cell cycle. There were two peaks in ODC

activity during the cell cycle; in late-G1/early-S and in late-S/G2. A decrease in ODC activity consistently occurred in mid-S phase. Maximum antizyme activity occurred in this phase of the cell cycle (VI). These results suggest that antizyme may be involved in the regulation of ODC activity during the cell cycle.

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5. REFERENCES

- Anderson E C, Petersen D F and Tobey R A (1970) *Biophys J* 10, 630.
- Andersson G (1977) *J Cell Physiol* 90, 329.
- Andersson G K A and Agrell I P S (1972) *Virchows Arch Abt B Zellpath* 11, 1.
- Andersson G and Heby O (1972) *J Natl Cancer Inst* 48, 165.
- Andersson G and Heby O (1977) *Cancer Res* 37, 4361.
- Anehus S, Emanuelsson H, Persson L, Sundler F, Scheffler I E and Heby O (1984 a) *Eur J Cell Biol* 35, 264.
- Anehus S, Linden M, Borg Å, Långström E and Heby O (1983) in *Advances in Polyamine Research*, Vol 4, Bachrach U, Kaye A and Chayen R (eds), Raven Press, New York, p 707.
- Anehus S, Pohjanpelto P, Baldetorp B, Långström E and Heby O (1984 b) *Mol Cell Biol* 4, 915.
- Atmar V J and Kuehn G D (1981) *Proc Natl Acad Sci USA* 78, 5518.
- Bachrach U (1973) *Function of Naturally Occurring Polyamines*, Academic Press, New York.
- Berger F G, Szymanski P, Read E and Watson G (1984) *J Biol Chem* 259, 7941.
- Bey P, Danzin C, Van Dorsselaer V, Mamont P, Jung M and Tardif C (1978) *J Med Chem* 21, 50.
- Bjerkvig R, Oredsson S M, Marton L J, Linden M and Deen D F (1983) *Cancer Res* 43, 1497.
- Branca A A and Herbst E J (1980) *Biochem J* 186, 925.
- Brosnan M E, Farrell R, Wilansky H and Williamson D H (1983) *Biochem J* 212, 149.
- Canellakis E S, Heller J S and Kyriakidis D A (1981) in *Advances in Polyamine Research*, Vol 3, Caldarera C M, Zappia V and Bachrach U (eds), Raven Press, New York, p 1.
- Canellakis E S, Heller J S, Kyriakidis D and Chen K Y (1978) in *Advances in Polyamine Research*, Vol 1, Campbell R A, Morris D R, Bartos D, Daves G D and Bartos F (eds), Raven Press, New York, p 155.
- Canellakis E S, Viceps-Madore D, Kyriakidis D A and Heller J S (1979) in *Current Topics in Cellular Regulation*, Vol 15, Horecker B L and Stadtman E R (eds), Academic Press, New York, p 155.
- Canellakis Z N and Theoharides I C (1976) *J Biol Chem* 251, 4436.
- Clark J L (1974) *Biochemistry* 13, 4668.

- Clark J L and Fuller J L (1976 a) *Biochem Biophys Res Commun* 73, 785.
- Clark J L and Fuller J L (1976 b) *Eur J Biochem* 67, 303.
- Cohen, S S (1971) *Introduction to the Polyamines*, Prentice-Hall, Englewood Cliffs, New Jersey.
- Commerford S L and Joel D D (1979) *Biochem Biophys Res Commun* 86, 112.
- Cooper E H, Bedford A J and Kenny T E (1975) *Adv Cancer Res* 21, 59.
- Dave C, Pathak S N and Porter C W (1978) in *Advances in Polyamine Research*, Vol 1, Campbell R A, Morris D R, Bartos D, Daves G D and Bartos F (eds), Raven Press, New York, p. 153.
- Dean P N (1980) *Cell Tissue Kinet* 13, 299.
- Delcros J-G, Roch A-M and Quash G (1984) *FEBS Lett* 171, 221.
- Dudley H W, Rosenheim O and Starling W W (1927 a) *Biochem J* 20, 1082.
- Dudley H W, Rosenheim O and Starling W W (1927 b) *Biochem J* 21, 97.
- Ehrlich P and Apolant H (1905) *Berl Klin Wochensh* 28, 871.
- Flangas A L (1974) *Prep Biochem* 4, 165.
- Folk J E (1980) *Annu Rev Biochem* 49, 517.
- Fong W F, Heller J S and Canellakis E S (1976) *Biochim Biophys Acta* 428, 456.
- Fujita K, Matsufuji S, Murakami Y and Hayashi S-I (1984) *Biochem J* 218, 557.
- Fujita K, Murakami Y and Hayashi S-I (1982) *Biochem J* 204, 647.
- Fujita S, Ashihara T and Fukuda M (1974) *Histochemistry* 40, 155.
- Glick D, von Redlich D, Juhos E T and McEwen C R (1971) *Exp Cell Res* 65, 23.
- Grabske R J, Lake S, Gledhill B L and Meistrich M L (1975) *J Cell Physiol* 86, 177.
- Hannonen P, Jänne J and Raina A (1972) *Biochim Biophys Acta* 289, 225.
- Heby O and Andersson G (1978) *Acta Path Microbiol Scand Sect A* 86, 17.
- Heby O, Andersson G and Gray J W (1978) *Exp Cell Res* 111, 461.
- Heby O and Emanuelsson H (1980) *Biochem Biophys Res Commun* 92, 75.
- Heby O, Gray J W, Lindl P A, Marton L J and Wilson C B (1976) *Biochem Biophys Res Commun* 71, 99.
- Heby O and Jänne J (1981) in *Polyamines in Biology and Medicine*, Morris D R and Marton L J (eds), Marcel Dekker, New York and Basel, p 243.

- Heby O, Marton L J, Wilson C B and Martinez H M (1975) J Cell Physiol 86, 511.
- Heby O, Sarna G P, Marton L J, Omine M, Perry S and Russell D H (1973) Cancer Res 33, 2959.
- Heller J S and Canellakis E S (1981) J Cell Physiol 107, 209.
- Heller J S, Chen K Y, Kyriakidis D A, Fong W F and Canellakis E S (1978) J Cell Physiol 96, 225.
- Heller J S, Fong W F and Canellakis E S (1976) Proc Natl Acad Sci USA 73, 1858.
- Heller J S, Kyriakidis D, Fong W F and Canellakis E S (1977) Eur J Biochem 81, 545.
- Hietala O A (1983) J Neurochem 40, 1174.
- Hofer K G, Prenskey W and Hughes W L (1969 a) J Natl Cancer Inst 43, 763.
- Hofer K G, Rosenoff S, Prenskey W and Hughes W L (1969 b) Nature 221, 576.
- Hölttä E (1975) Biochim Biophys Acta 399, 420.
- Hölttä E, Hannonen P, Pispä J and Jänne J (1973) Biochem J 136, 669.
- Isomaa V V, Pajunen A E I, Bardin C W and Jänne O A (1983) J Biol Chem 258, 6735.
- Jänne J, Pösö H and Raina A (1978) Biochim Biophys Acta 473, 241.
- Jänne J and Raina A (1968) Acta Chem Scand 22, 1349.
- Jänne J and Williams-Ashman H G (1971) J Biol Chem 246, 1725.
- Kallio A (1978) Acta Chem Scand B 32, 759.
- Kallio A, Löfman M, Pösö H and Jänne J (1977 a) FEBS Lett 79, 195.
- Kallio A, Pösö H, Scalabrino G and Jänne J (1977 b) FEBS Lett 73, 229.
- Kameji T, Fujita K, Noguchi T, Takiguchi M, Mori M, Tatibana M and Hayashi S-I (1984) Eur J Biochem 144, 35.
- Kameji T, Murakami Y, Fujita K and Hayashi S-I (1982) Biochim Biophys Acta 717, 111.
- Kameji T, Murakami Y, Fujita K, Noguchi T and Hayashi S (1981) Med Biol 59, 296.
- Keng P C, Li C K N and Wheeler K T (1980) Cell Biophys 2, 193.
- Kitani T and Fujisawa H (1983) J Biol Chem 258, 235.
- Kitani T and Fujisawa H (1984) J Biol Chem 259, 10036.

- Kontula K K, Torkkeli T K, Bardin C W and Jänne O A (1984) *Proc Natl Acad Sci USA* 81, 731.
- Ladenburg A and Abel J (1888) *Ber Dtsch Chem Ges* 21,758.
- Lesiewicz J and Goldsmith L A (1983) *J Invest Dermatol* 80, 97.
- Lindahl P E (1948) *Nature* 161, 648.
- Luk G D, Goodwin G, Marton L J and Baylin S B (1981) *Proc Natl Acad Sci USA* 78, 2355.
- Mamont P S, Danzin C, Wagner J, Siat M, Joder-Ohlenbusch A-M and Claverie N (1982) *Eur J Biochem* 123, 499.
- Mamont P S, Duchesne M-C, Joder-Ohlenbusch A-M and Grove J (1978) in *Enzyme-Activated Irreversible Inhibitors*, Seiler N, Jung M J and Koch-Weser J (eds), Elsevier/North Holland Biochemical Press, Amsterdam, p 43.
- McCann P P (1980) in *Polyamines in Biomedical Research*, Gaugas J M (ed), John Wiley & Sons, New York, p. 109.
- McCann P P, Tardif C and Mamont P S (1977) *Biochem Biophys Res Commun* 75, 948.
- Metcalf B W, Bey P, Danzin C, Jung M J, Casara P and Vever J P (1978) *J Am Chem Soc* 100, 2551.
- Nemoto O, Aoyagi T and Miura Y (1984) *J Invest Dermatol* 83, 257.
- Obernader M F and Prouty W F (1977) *J Biol Chem* 252, 2866.
- Oredsson S, Anehus S and Heby O (1980) *Biochem Biophys Res Commun* 94, 151.
- Pegg A E, Conover C and Wrona A (1978) *Biochem J* 170, 651.
- Pegg A E and McCann P P (1982) *Am J Physiol* 243, C 212.
- Pegg A E and McGill S M (1978) *Biochem Pharmacol* 27, 1625.
- Pegg A E, Pösö H, Shuttleworth K and Bennett R A (1982) *Biochem J* 202, 519.
- Pegg A E and Williams-Ashman H G (1968) *Biochem J* 108, 533.
- Pegg A E and Williams-Ashman H G (1969) *J Biol Chem* 244, 682.
- Pegg A E and Williams-Ashman H G (1981) in *Polyamines in Biology and Medicine*, Morris D R and Marton L J (eds), Marcel Dekker, New York, p 3.
- Persson L (1981 a) *Acta Chem Scand B* 35, 451.
- Persson L (1981 b) *Acta Chem Scand B* 35, 737.
- Persson L (1982) *Acta Chem Scand B* 36, 685.

- Persson L and Rosengren E (1979) *Acta Chem Scand B* 33, 537.
- Pett D M and Ginsberg H S (1968) *Fed Proc* 27, 615.
- Piik K, Pösö H and Jänne J (1978) *FEBS Lett* 89, 307.
- Piik K, Rajamäki P, Guha S K and Jänne J (1977) *Biochem J* 168, 379.
- Pohjanpelto P, Virtanen I and Hölttä E (1981) *Nature* 293, 475.
- Porter C W, Dave C and Mihich E (1981) in *Polyamines in Biology and Medicine*, Morris D R and Marton L J (eds), Marcel Dekker, New York and Basel, p 407.
- Pösö H and Jänne J (1976) *Biochem Biophys Res Commun* 69, 885.
- Rosenheim O (1924) *Biochem J* 18, 1253.
- Russell D H (1980) *Pharmacology* 20, 117.
- Russell D H (1981) *Biochem Biophys Res Commun* 99, 1167.
- Russell D H and Snyder S H (1968) *Proc Natl Acad Sci U S A* 60, 1420.
- Russell D H and Snyder S H (1969) *Mol Pharmacol* 5, 253.
- Sano Y, Dean D F, Oredsson S M and Marton L J (1984) *Cancer Res* 44, 577.
- Seely J E and Pegg A E (1983 a) *J Biol Chem* 258, 2496.
- Seely J E and Pegg A E (1983 b) *Biochem J* 216, 701.
- Seely J E, Pösö H and Pegg A E (1982) *Biochemistry* 21, 3394.
- Seiler N (1970) *Meth Biochem Anal* 18, 259.
- Steen H B and Lindmo T (1978) *Cell Tissue Kinet* 11, 69.
- Sunkara P S, Rao P N and Nishioka K (1977) *Biochem Biophys Res Commun* 74, 1125.
- Theoharides T C and Canellakis Z N (1976) *J Biol Chem* 251, 4436.
- Van Leeuwenhoek A (1678) *Phil Trans Roy Soc* 12, 1040.

Inhibition of ornithine decarboxylase activity and cell growth by diamines. A comparison between the effects of two homologs, 1,3-diaminopropane and 1,4-diaminobutane (putrescine).

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Running head: Effects of diamines on cell growth

Key words: 1,3-diaminopropane, putrescine, α -difluoromethylornithine, polyamines, ornithine decarboxylase, cell size, cell proliferation, cell cycle, flow cytometry, DNA histogram

The ornithine decarboxylase (ODC) activity of Ehrlich ascites tumor cells was almost completely inhibited by treatment with either putrescine (10 mM) or 1,3-diaminopropane (5 mM). 1,3-Diaminopropane treatment eradicated the cellular content of putrescine and reduced that of spermidine and spermine. Putrescine treatment caused a dramatic increase in cellular putrescine content, and a temporary decrease in spermidine and spermine content. Despite the fact that 1,3-diaminopropane and putrescine inhibited the ODC activity more effectively than did α -difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ODC, they were considerably less antiproliferative in action. However, as compared to DFMO the diamines were less effective in reducing the total polyamine (putrescine + spermidine + spermine) content of the cells.

Ornithine decarboxylase (ODC; EC 4.1.1.17), is a key enzyme in polyamine synthesis and cell growth (1,2). It has a remarkably short half-life (3-5) and is subject to many control mechanisms (2,6). Upon enzyme-activated irreversible inhibition of the ODC activity by treatment with α -difluoromethylornithine (DFMO), cells are depleted of their putrescine and spermidine content and, as a consequence, cease to proliferate (2). The ODC activity is also inhibited by treatment with di- and polyamines (for references, see (2)). Using a competitive radioimmunoassay procedure, Seely and Pegg (4,5) have recently demonstrated that the ODC activity and the amount of enzyme protein decrease in parallel as a result of diamine treatment. Certain diamines, particularly 1,4-diaminobutane (putrescine) and its lower homolog 1,3-diaminopropane, have been shown to induce the synthesis and/or release of a 26,000 molecular weight ODC inhibitory protein, termed antizyme (6-10). This protein may be involved in the rapid degradation of ODC.

These findings raise the question whether 1,3-diaminopropane, by inhibiting the ODC activity, may be used, in a similar manner as DFMO, to deplete the cellular polyamine content and thereby exert an anti-proliferative effect. Although it has been reported that administration of 1,3-diaminopropane inhibits the incorporation of (^3H)-thymidine into DNA (11-14), the relationship between polyamine depletion and the antiproliferative effect has not been analyzed in detail.

The present study was designed to 1) substantiate the effects of treatment with 1,3-diaminopropane on polyamine metabolism and cell growth, and 2) compare the effects of 1,3-diaminopropane with those of the higher homolog putrescine, which is a product of the ODC-catalyzed reaction and therefore should counteract polyamine depletion and

growth inhibition. In these experiments the concentrations of putrescine and 1,3-diaminopropane were carefully chosen; such that they almost completely eradicated the ODC activity and yet did not produce any apparent cytotoxicity.

MATERIALS AND METHODS

Cell Culture

A hyperdiploid subline of the Ehrlich ascites tumor was grown in suspension culture in a medium supplemented with 10% fetal calf serum (15). At time 0, 1.0×10^7 plateau phase cells were suspended in 100 ml of growth medium and seeded in a 150 cm² Costar tissue culture flask. In addition, the same number of cells was seeded in 100 ml of growth medium containing putrescine (0.01, 1 or 10 mM) (Fluka), 1,3-diaminopropane (5 mM) (EGA-Chemie) or DFMO (5 mM). Stock solutions of the diamines and DFMO were made up at 100X concentrations in complete culture medium. Before addition to the cell cultures the solutions were adjusted to pH 7.4 and sterilized by passage through 0.20 μ m pore size filters (Flow). A Coulter counter (Industrial Model D) was used to determine the cell number. Every 12 hr, samples were removed for cell counting, ODC assay, polyamine analysis, cell size distribution analysis and cell cycle distribution analysis. Cells intended for biochemical analyses were harvested by centrifugation at $1,000 \times g$ for 10 min at 4°C. The pellets were stored at -70°C.

Cells that had been subjected to putrescine treatment were washed once in a large volume (10 ml) of phosphate-buffered saline (pH 7.2) in order to remove excess putrescine from the cell pellet.

This wash reduced the amount of putrescine associated with the cell pellet without affecting the spermidine and spermine content of the cells. The putrescine content of these cells has to be considered an approximation, however, because we cannot exclude the possibility that some intracellular putrescine may have been lost during the wash. Inasmuch as there were negligible amounts of polyamines in the medium, 1,3-diaminopropane-treated cells and untreated control cells were not washed.

ODC Assay

Cells were disrupted by sonication in ice-cold 10 mM Tris-HCl buffer (pH 7.2) containing 5 mM dithiothreitol, 0.05 mM pyridoxal 5'-phosphate and 0.5 mM Na₂EDTA. The ODC activity was assayed for in the presence of a saturating (1 mM) L-ornithine concentration essentially as described by Jänne and Williams-Ashman (16).

Polyamine Analysis

Cells were disrupted by sonication in ice-cold 0.2 M perchloric acid. After 1 hr on ice, the homogenate was centrifuged at 1,000 x g for 10 min at 4°C. A 200 µl aliquot of the supernatant was dansylated and analyzed by thin-layer chromatography (17,18).

Cell Size Distribution Analysis

Approximately 1×10^6 cells, grown in the absence or presence of 10 mM putrescine or 5mM 1,3-diaminopropane for 96 hr, were suspended in 100 ml of saline containing 1% formaldehyde. The cell size distribution was analyzed using a Coulter counter (Industrial Model D).

Flow Cytometric Analysis

Cells were harvested by centrifugation at $500 \times g$ for 5 min, fixed in absolute ethanol ($1-2 \times 10^6$ cells/ml) at -20°C and analyzed for their cell cycle distribution by flow cytometry. Prior to analysis, the cells were treated with pepsin, RNase and Nonidet P40 and stained with propidium iodide as previously described (19). The stained cell nuclei were analyzed using a Cytofluorograph 50-H (Ortho Instruments).

RESULTS

Figure 1 shows the effects of treatment with various putrescine concentrations on Ehrlich ascites tumor cell growth in culture. Inhibition of cell growth exerted by putrescine was dose dependent. The reduction in growth rate, caused by putrescine concentrations up to 10 mM, did not exceed 21% (Fig. 1, 3A).

The effects of treatment with the same putrescine concentrations on the cellular ODC activity are shown in Figure 2. In control cells three peaks of ODC activity were observed during the 96-hr growth period; at 3, 10 and 24 hr after seeding. Putrescine treatment reduced all three increases in ODC activity in a dose dependent manner. A 10 mM putrescine concentration was required to completely prevent the increases in ODC activity.

The effects of treatment with 5 mM 1,3-diaminopropane and 10 mM putrescine, on cell growth and polyamine metabolism are compared in Figure 3. These concentrations were required to almost completely inhibit (by 96-100%) the ODC activity (Fig. 3B,G). Both diamines

exerted a similar, approximately 20%, inhibitory effect on tumor cell growth rate (Fig. 3A,F). Administration of putrescine caused a rapid intracellular accumulation of this diamine (Fig. 3C). Conversely, 1,3-diaminopropane treatment eradicated the cellular putrescine content within 12 hr (Fig. 3H). In cells treated with putrescine, the spermidine content decreased during the first 24 hr and returned to the control level by 36 hr (Fig. 3D). Treatment with 1,3-diaminopropane resulted in a continuous decrease in the cellular spermidine content (Fig. 3I). Treatment with either diamine first resulted in a decrease in the cellular spermine content, but 2 days after seeding the control level was reached (Fig. 3E,J).

Putrescine and 1,3-diaminopropane treatment caused a significant increase in average cell size (Fig. 4). Nevertheless, flow cytometric analyses of cells treated with putrescine or 1,3-diaminopropane revealed only a slight change or no change at all in cell cycle distribution (Fig. 5). A slight increase in the G2 phase fraction and a corresponding decrease in G1 and S was consistently observed in putrescine-treated cells (Fig. 5B).

Table 1 shows the effects of treatment (72 hr) with putrescine and 1,3-diaminopropane, compared to that of DFMO, on total polyamine (putrescine + spermidine + spermine) content and cell proliferation. Only 1,3-diaminopropane and DFMO treatment reduced the total polyamine content. DFMO was more effective than 1,3-diaminopropane at the concentration used (5mM). Putrescine and 1,3-diaminopropane inhibited cell growth to approximately the same extent. DFMO exerted a significantly greater antiproliferative effect (Table 1).

DISCUSSION

In agreement with many previous reports (for references, see (2)) the present study shows that treatment with the diamines putrescine and 1,3-diaminopropane inhibits the cellular ODC activity. Thus, a 96-100% inhibition of the ODC activity in Ehrlich ascites tumor cells was achieved by treatment with 10 mM putrescine or 5 mM 1,3-diaminopropane. Studies on Chinese hamster ovary cells, Ehrlich ascites tumor cells, diet-stimulated and regenerating rat liver, show that diamine-induced ODC inhibition causes a reduction in the incorporation of (^3H)thymidine into DNA (11-14,20) and a lower mitotic index (14,20).

In this report we assess the antiproliferative effects exerted by putrescine and 1,3-diaminopropane, and analyze whether they are due to interference with any particular phase of the cell cycle. The diamine concentrations were chosen such that they produced a total eradication of the ODC activity, yet did not show any apparent cytotoxic effect. Even though the cells were incubated with rather high diamine concentrations and for long time periods in the presence of fetal calf serum, there appeared to be no toxicity due to serum oxidases inasmuch as treatment with aminoguanidine, a potent inhibitor of diamine oxidase, failed to reduce the antiproliferative effect of putrescine (21). That fetal calf serum does not oxidize putrescine is also suggested by the fact that all radioactive putrescine was recovered intact after a 4 hr incubation with 100 % fetal calf serum (22).

Putrescine and 1,3-diaminopropane exerted a similar antiproliferative action. 1,3-Diaminopropane treatment did not change the cell cycle distribution while putrescine treatment caused a slight accumu-

lation of cells in the G2 phase. Nevertheless, treatment with either diamine resulted in a cell size increase. Since there was no major change in cell cycle distribution in diamine-treated cells it appears that all cell cycle phases are prolonged and that cell enlargement is due to unbalanced growth. This is at variance with the results of a previous study in which we demonstrated that putrescine treatment of the Ehrlich ascites tumor in vivo caused an accumulation of cells in the S phase, mainly due to a decrease in the rate of DNA synthesis (20). Furthermore, the results of Sunkara et al (11) suggest that 1,3-diaminopropane treatment causes an accumulation of CHO cells in the S phase. The more pronounced cell cycle effects in these previous studies may be due to the fact that the diamines were given at higher, possibly toxic, concentrations.

Despite the increase in cellular putrescine content during treatment with exogenous putrescine, the spermidine and spermine content decreased initially, as compared to the control. The decrease in spermidine content may be due to the fact that putrescine, like 1,3-diaminopropane, inhibits S-adenosylmethionine decarboxylase (SAMDC) at high concentrations (23). The decrease in spermine content is probably due to the fact that putrescine acts as a competitive inhibitor of spermine synthase. Thus, putrescine competes with spermidine for the active site of the enzyme (24).

1,3-Diaminopropane treatment totally inhibited putrescine synthesis by eradicating the ODC activity. The substantial decrease in spermidine content resulting from this treatment may be due partly to lack of putrescine (by 12 hr) and partly to a direct inhibitory effect of 1,3-diaminopropane on the SAMDC activity (23). The decrease in spermine content after 1,3-diaminopropane administration, is unexpec-

ted when considering the effects of DFMO treatment. Thus treatment with DFMO very effectively depletes the putrescine and spermidine content and increases the spermine content of Ehrlich ascites tumor cells (15). Since putrescine is a competitive inhibitor of spermine synthase, putrescine depletion relieves this inhibition and the spermine content increases. That 1,3-diaminopropane instead causes a decrease in cellular spermine content despite the depletion of putrescine therefore suggests that 1,3-diaminopropane inhibits spermine synthase in a similar manner as does putrescine (24). An inhibitory effect of 1,3-diaminopropane on the activity of spermine synthase has been observed in vitro (25).

Even if 10 mM putrescine inhibits the cellular ODC activity completely there should be no reduction in putrescine, spermidine and spermine content since putrescine is a product of the ODC-catalyzed reaction. Nevertheless, putrescine treatment reduced the spermidine and spermine content, possibly due to inhibition of the SAMDC and spermine synthase activities at this rather high concentration (10 mM). The antiproliferative effect of putrescine treatment therefore seems to be a result of the decrease in spermidine and spermine content. At a lower (1 mM) concentration putrescine completely reverses the antiproliferative effect of DFMO-induced putrescine and spermidine deficiency (21, 26). There is a corresponding decrease in the cellular content of spermidine and spermine during the initial phase of 1,3-diaminopropane treatment, i.e. when the antiproliferative effect is established. The fact that 1,3-diaminopropane treatment in addition causes putrescine depletion but is not more inhibitory to cell proliferation (than is putrescine treatment), suggests that 1,3-diaminopropane can partly replace putrescine in its cellular function. In expe-

riments in which Ehrlich ascites tumor cells were treated with a combination of DFMO and 1,3-diaminopropane, there was a further decrease in spermine content (compared to that observed in cells treated with DFMO alone) but no major potentiation of the antiproliferative effect (21, 27). A possible interpretation of this, which is consistent with the present data, is that 1,3-diaminopropane partly replaces putrescine in its function at the same time as it blocks spermine synthesis. In fact, 1,3-diaminopropane can partly replace the role played by putrescine in rat hepatoma cell growth (26). That DFMO exerts a more potent antiproliferative effect than does putrescine and 1,3-diaminopropane may be a consequence of the depletion of putrescine (in addition to that to spermidine) with no diamine substitute present. Furthermore, DFMO-treated cells have a lower content of total polyamines (putrescine + spermidine + spermine) (Table 1).

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REFERENCES

1. Pegg AE, Williams-Ashman HG: Polyamines in Biology and Medicine (Morris DR, Marton LJ, eds), Marcel Dekker Inc, New York, p 3, 1981.
2. Heby O, Jänne J: *ibid*, p 243, 1981.
3. Russell DH, Snyder SH: *Mol Pharmacol* 5:253, 1969.
4. Seely JE, Pegg AE: *J Biol Chem* 258:2496, 1983.
5. Seely JE, Pegg AE: *Biochem J* 216:701, 1983.
6. Canellakis ES, Viceps-Madore D, Kyriakidis DA, Heller JS: *Curr Topics Cell Reg* 15:155, 1979.
7. Heller JS, Fong WF, Canellakis ES: *Proc Natl Acad Sci USA* 73:1858, 1976.
8. Kallio A, Löfman M, Pösö H, Jänne J: *Biochem J* 177:63, 1979.
9. McCann PP, Tardif C, Mamont PS: *Biochem Biophys Res Commun* 75:948, 1977.
10. McCann PP, Tardif C, Pegg AE, Diekema K: *Life Sci* 26:2003, 1980.
11. Sunkara PS, Rao PN, Nishioka K: *Biochem Biophys Res Commun* 74:1125, 1977.
12. Kallio A, Pösö H, Jänne J: *Biochim Biophys Acta* 479:345, 1977.
13. Alhonen-Hongisto L, Kallio A, Pösö H, Jänne J: *Biochim Biophys Acta* 564:473, 1979.
14. Kameji T, Murakami Y, Hayashi S: *J Biochem* 86:191, 1979.
15. Oredsson S, Anehus S, Heby O: *Biochem Biophys Res Commun* 94:151, 1980.
16. Jänne J, Williams-Ashman HG: *J Biol Chem* 246:1725, 1971.
17. Seiler N: *Meth Biochem Anal* 18:259, 1970.
18. Heby O, Sarna GP, Marton LJ, Omine M, Perry S, Russell DH: *Cancer Res* 33:2959, 1973.

19. Anehus S, Pohjanpelto P, Baldetorp B, Långström E, Heby O: *Mol Cell Biol* 4:915, 1984.
20. Linden M, Andersson G, Heby O: *Int J Biochem* 12:387, 1980.
21. Heby O, Anehus S, Linden M, Oredsson S: *Advances in Polyamine Research* (Caldarera CM, Zappia V, Bachrach U, eds) Raven Press, New York, p 357, 1981.
22. Heller JS, Chen KY, Kyriakidis DA, Fong WF, Canellakis ES: *J Cell Physiol* 96:225, 1978.
23. Piik K, Rajamäki P, Guha SK, Jänne J: *Biochem J* 168:379, 1977.
24. Hannonen P, Jänne J, Raina A: *Biochim Biophys Acta* 289:225, 1972.
25. Hibasami H, Pegg AE: *Biochem Biophys Res Commun* 81:1398, 1978.
26. Mamont PS, Duchesne M-C, Joder-Ohlenbusch A-M, Grove J: *Enzyme-activated irreversible inhibitors* (Seiler N, Jung MJ, Koch-Weser J, eds), Elsevier/North-Holland Biomedical Press, Amsterdam, p 43, 1978.
27. Oredsson SM, Anehus S, Heby O: *Mol Cell Biochem* 64: 163, 1984.
28. Dean PN: *Cell Tissue Kinet* 13:299, 1980.

Table 1. Effects of ODC inhibition on total polyamine content and proliferation of Ehrlich ascites tumor cells.

Treatment*	Cell number	Total polyamine content
		(% of 72 hr control)
Putrescine (10 mM)	63	123
1,3-Diaminopropane (5 mM)	69	72
DFMO (5 mM)	36	19

* Putrescine, 1,3-diaminopropane and DFMO were added to the cell cultures at the time of seeding and the cells were harvested after 72 hr of treatment.

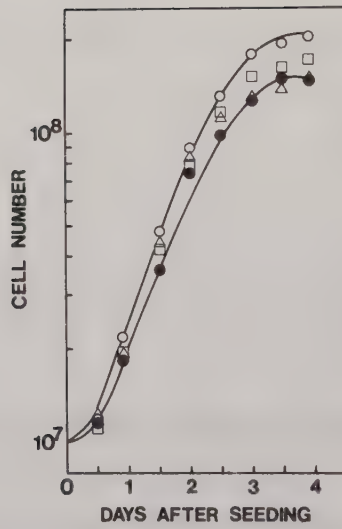


Fig. 1. Effect of putrescine on Ehrlich ascites tumor cell growth. Putrescine was added at time 0 to a final concentration of 0.01 mM (□), 1.0 mM (△) or 10 mM (●). Untreated control culture (○).

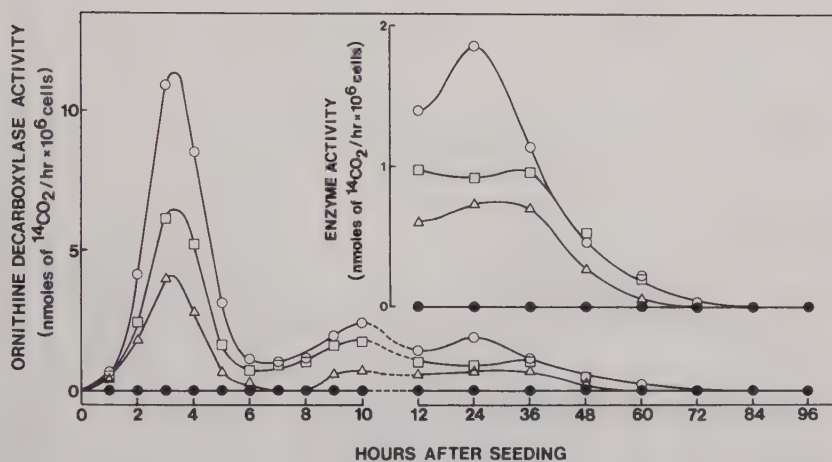


Fig. 2. Effect of putrescine on the activity of ornithine decarboxylase in Ehrlich ascites tumor cells. Putrescine was added at time 0 to a final concentration of 0.01 mM (□), 1.0 mM (△), or 10 mM (●). Untreated control culture (○). To elucidate the effects occurring after 12 hr of growth, the ordinate was expanded 5-fold (inset).

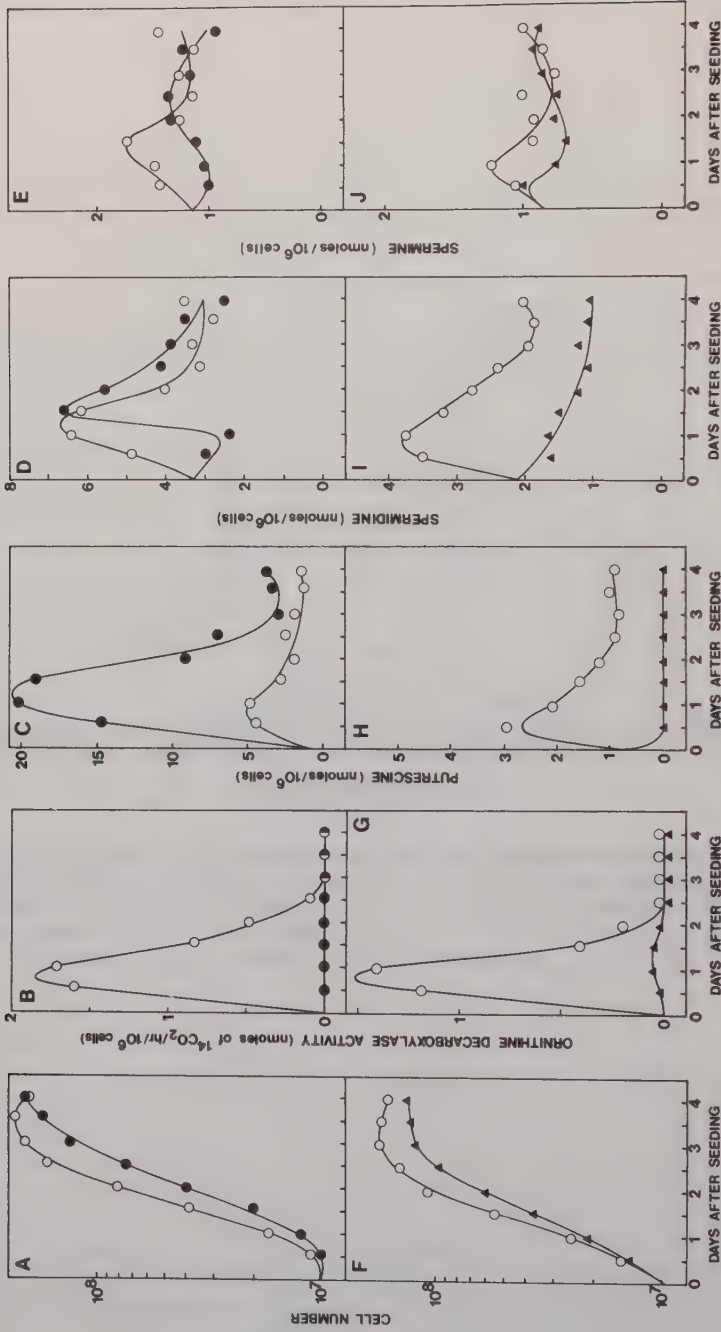


Fig. 3. Effects of 10 mM putrescine (A-E) and 5 mM 1,3-diaminopropane (F-J) on Ehrlich ascites tumor cell growth and polyamine synthesis and content. Putrescine (●) and 1,3-diaminopropane (▲) were added at time 0. Untreated control cultures (○).

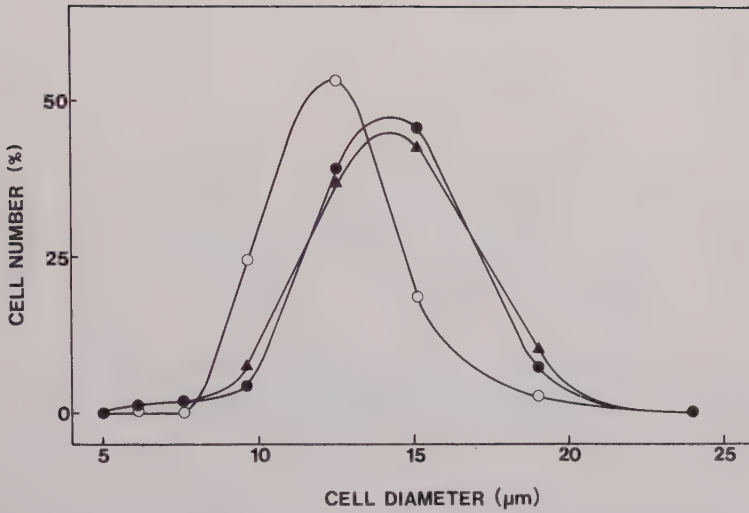


Fig. 4. Effect of 10 mM putrescine and 5 mM 1,3-diaminopropane on cell size distribution of Ehrlich ascites tumor cells. Putrescine (●) and 1,3-diaminopropane (▲) were added at time 0 and the cells were harvested 96 hr later. Untreated 96-hr control culture (○). Individual data points represent the percentage of cells exhibiting a certain diameter (in each of the three cell populations).

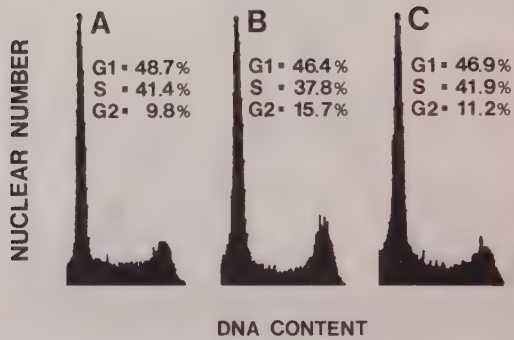


Fig. 5. Effect of 10 mM putrescine and 5 mM 1,3-diaminopropane on the cell cycle distribution of Ehrlich ascites tumor cells. Putrescine (B) and 1,3-diaminopropane (C) were added at time 0 and the cells were harvested 96 hr later. Untreated 96 hr control culture (A). The DNA histograms were obtained by flow cytometry and the percentage of cells in G1, S and G2 was calculated essentially as described by Dean (28).

Cell Cycle Phase-Dependent Induction of Ornithine Decarboxylase-
-Antizyme¹.

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Running title: ODC-Antizyme and the Cell Cycle

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The activities of ornithine decarboxylase (ODC) and ODC inhibitory protein (ODC-antizyme) were studied in Ehrlich ascites tumor cells separated according to their position in the cell cycle by centrifugal elutriation. Release and/or synthesis of ODC-antizyme was induced by putrescine treatment (each mouse received an i.p. injection of 25 μ moles of putrescine at 0, 1, 2 and 3 hr after tumor transplantation). Tumor cells obtained from putrescine-treated and control mice at 4 hr after transplantation were separated into fractions representing all phases of the cell cycle. The cell cycle distribution of the tumor cells in each fraction was determined by flow cytometry.

In control tumor cells the ODC activity exhibited two maxima; in late-G1/early-S and in late-S/G2. A marked decrease in ODC activity was observed in mid-S phase. This decrease coincided with maximum ODC-antizyme activity (revealed by putrescine treatment), suggesting that ODC-antizyme is involved in the regulation of ODC activity during the cell cycle.

The activity of ornithine decarboxylase (ODC; EC 4.1.1.17), a key enzyme in polyamine synthesis and cell growth, is almost non-detectable in quiescent cells. After exposure to various stimuli, however, the ODC activity increases dramatically, within just a few hours (Jänne et al., 1978; McCann, 1980). ODC is rapidly synthesized and degraded and its half-life may be as short as 5 min (Hogan et al., 1974; Russell, 1980; Pegg and Williams-Ashman, 1981).

The ODC activity may be regulated at the post-translational level by modifications such as phosphorylation (Atmar and Kuehn, 1981) and transglutamination (Folk, 1980; Russell, 1981; Delcros et al., 1984), and by interaction with ODC-inhibitory proteins (Canellakis et al., 1979; Kaye and Yariv, 1983) and ODC-activating proteins (Canellakis et al., 1981; Fujita et al., 1982). Altered binding of pyridoxal 5'-phosphate may also be a means of regulating the ODC activity (Clark and Fuller, 1976; Mitchell, 1981). Most of the evidence for these various regulatory mechanisms comes from in vitro experiments. Therefore it is unclear to what extent they regulate the ODC activity in vivo.

Various di- and polyamines, particularly putrescine, have been shown to decrease the ODC activity and simultaneously to induce the release and/or synthesis of an ODC-inhibitory protein termed ODC-antizyme (Heller et al., 1976; McCann et al., 1977; Pegg et al., 1978). ODC-antizyme, a 19-22,000 molecular weight protein (Kitani and Fujisawa, 1984), inhibits ODC by noncompetitive binding (Heller et al., 1976). The complex formed can be dissociated with high salt concentrations and each component can be recovered in its active form (Heller et al., 1976; McCann et al., 1977). Recently, it has been suggested that the physiological role of ODC-antizyme could be to make ODC available to a particular fast-acting proteolytic system (Heller et al., 1976; Seely and Pegg, 1983).

In the present work, we have studied the activity of ODC-antizyme in Ehrlich ascites tumor cells in an attempt to determine whether ODC-antizyme participates in the regulation of ODC activity during the cell cycle. The ODC activity was stimulated by i.p. transplantation of tumor cells in plateau phase growth into new host mice. Release and/or synthesis of ODC-antizyme was induced by putrescine treatment (repeated i.p. injections of 25 μ moles/hr per mouse) of tumor-bearing mice (Linden et al., 1980). The ascites tumor cells were collected 4 hr after transplantation and separated according to their size using a centrifugal elutriation technique. Tumor cell fractions were then analyzed for their DNA distribution, and for their ODC or ODC-antizyme activities. The changes observed in the latter activities suggest that ODC-antizyme may be involved in the regulation of ODC activity during the Ehrlich ascites tumor cell cycle in vivo.

MATERIALS AND METHODS

Tumor cells

Hyperdiploid Ehrlich ascites tumor cells were grown in the peritoneal cavity of 6-week-old males of inbred NMRI mice. The tumor cells were routinely transplanted every 12th day to new hosts (Linden et al., 1980). In the present study, mice were given an i.p. inoculation of 5×10^7 cells (suspended in 0.6 ml of phosphate-buffered (pH 7.2) saline; PBS) obtained from a 12-day-old ascites tumor.

ODC and ODC-antizyme induction

The ODC activity was stimulated by i.p. transplantation of plateau phase (12-day-old) tumor cells into new hosts. In order to induce the release and/or synthesis of ODC-antizyme the new hosts were given 4 i.p. injections of putrescine (25 μ moles in 0.2 ml of PBS/mouse) at 0, 1, 2 and 3 hr after tumor transplantation. Control mice were given 4 i.p. injections of 0.2 ml of PBS at the same intervals. The tumor cells were then collected at 4 hr after transplantation by rinsing the peritoneal cavity with 10 ml of ice-cold PBS. This experimental protocol was used because maximum ODC activity is induced 4 hr after tumor transplantation and maximum ODC-antizyme activity is induced after 4 hr of treatment with putrescine (Linden et al., unpublished). All experiments were repeated 4 times.

Cell separation

Ehrlich ascites tumor cells, pooled from 3-5 mice at 4 hr after transplantation, were separated according to size by centrifugal elutriation using a modification (Anehus et al., 1983) of the method described by Grabske et al., (1975). Briefly, $1-1.5 \times 10^8$ tumor cells, from putrescine-treated or control mice, were suspended in 5-10 ml of ice-cold PBS and injected into the separation chamber of a Beckman JE-6 rotor. Ice-cold PBS was pumped through the elutriation system at a constant flow-rate of 20 ml/min. A rotor speed of 2,700 rpm was used during loading of the cells. The first two fractions containing 250 ml each were discarded. Then the rotor speed was reduced by 100 rpm and a 250 ml fraction was collected. By step-wise (100 rpm/step) reduction of the rotor speed, 6-7 additional 250 ml fractions were collected.

All fractions were centrifuged at 1,000 $\times$ g for 15 min at 4 $^{\circ}$ C in an International centrifuge. The cell pellets were

resuspended in 5-10 ml PBS and the total cell number in each fraction was calculated after counting an aliquot in a Bürker chamber. The cells in each fraction were then processed for flow cytometric analysis and for assay of ODC or ODC-antizyme activities.

Cell cycle analysis

Approximately 1×10^6 cells from each fraction were stained with propidium iodide and analyzed for their DNA content using a Cytofluorograph System 50-H (Ortho Instruments, Westwood, Ma) as previously described (Anehus et al., 1984). The percentage of cells in each phase of the cell cycle was calculated from the DNA distributions essentially as described by Dean (1980).

ODC and ODC-antizyme assays

Cells from all fractions were analyzed for ODC or free ODC-antizyme activities. ODC activity was determined according to the method of Jänne and Williams-Ashman (1971). ODC-antizyme activity was analyzed by measuring the inhibition of ODC activity in vitro according to the method of Heller et al. (1976) as modified by Linden et al. (1980). One unit of ODC-antizyme activity is defined as the amount which inhibits one unit of ODC activity, i.e. 1 nmole of $\text{CO}_2/\text{hr}/\text{mg}$ protein.

The cellular protein content was analyzed as described by Lowry et al. (1951).

RESULTS

Ehrlich ascites tumor cells were separated into fractions according to their size using a centrifugal elutriation technique. Figures 1A and 2A show the cellular DNA distributions in consecutive fractions obtained by elutriation of tumor cells from control and putrescine-treated mice, respectively. The percentage of cells in the G1, S and G2 phases of the cell cycle, was estimated for each elutriated fraction (Figures 1B and 2B). Putrescine treatment of tumor-bearing mice during the first 4 hours after transplantation did not affect the cell cycle distribution in the tumor cell population (Anehus et al, 1983). The tumor cell fractions derived from control mice were analyzed for ODC activity (Figure 1) and those derived from putrescine-treated mice (which showed no ODC activity) were analyzed for ODC-antizyme activity (Figure 2). To facilitate a comparison between cell cycle-related changes in ODC and ODC-antizyme activities, we have chosen one set of experiments (out of 4) (Figs. 1 and 2) in which the corresponding cell fractions were almost identical with regard to cell cycle distribution (cf. Figs. 1A and 2A).

Figure 1C shows that fractions in which a majority of the cells are in late-G1/early-S or in late-S/G2 exhibit maximum ODC activities. The fraction in which most cells are in mid-S shows a decrease in ODC activity. Notably, maximum ODC-antizyme activity was detected in the corresponding cell fraction (Fig. 2C). The same temporal relationship between ODC and ODC-antizyme activity was borne out in all 4 sets of experiments.

DISCUSSION

The complex nature of ODC regulation has intrigued many investigators and a great number of modification mechanisms have been described, mainly on the basis of in vitro experiments (Clark and Fuller, 1976; Canellakis et al., 1979, 1981; Folk, 1980; Atmar and Kuehn, 1981; Mitchell, 1981; Russell, 1981; Fujita et al., 1982; Kaye and Yariv, 1983; Delcros et al., 1984). Thus far, however, the relevance of these various mechanisms for the in vivo regulation of ODC activity remains poorly understood. In the present study we have attempted to determine whether ODC-antizyme, one of the negative effectors (Heller et al., 1976; McCann et al., 1977; Pegg et al., 1978), plays a role in the regulation of ODC activity during the cell cycle.

In a previous study we showed that repeated i.p. injections of putrescine (25 μ moles/hr per mouse) into ascites tumor-bearing mice caused an almost complete eradication of the ODC activity in the tumor cells, which paralleled the appearance of an endogenous ODC inhibitor (Linden et al., 1980). The ODC inhibitory activity depended on the dose of putrescine, but was not caused by product (putrescine) inhibition. Instead the inhibition of ODC was shown to be the result of complex formation with a macromolecular inhibitor. This inhibitor was separated from ODC by gel filtration (Sephadex G-75 Superfine) in the presence of 250 mM NaCl. Its elution pattern was similar to that of ODC-antizyme (McCann et al., 1977).

During the Ehrlich ascites tumor cell cycle there are two peaks in ODC activity, in late-G1/early-S and in late-S/G2 (Fig. 1). Similar changes in ODC activity have been described for other cell types (Heby et al., 1976; Heby and Jänne, 1981). If ODC-antizyme is involved in the regulation of the ODC activity, one would expect to

find maximum antizyme activity in cells where the ODC activity is suppressed. To determine whether the decrease in ODC activity occurring in mid-S phase might be due to the appearance of ODC-antizyme, the following experiment was performed.

ODC-antizyme activity was induced in exponentially growing Ehrlich ascites tumor cells by repeated i.p. injections of putrescine. After 4 hr of treatment, when the ODC activity had been eradicated and the antizyme activity was maximal, the cells were separated by centrifugal elutriation. This technique makes it possible to separate asynchronously growing cells according to their size, which is a function of their position in the cell cycle. This implies that all cells, irrespective of their position in the cell cycle, have been exposed to identical experimental conditions, in the present case, to the same dose and time of exposure to putrescine.

Putrescine-treated cells exhibited their highest ODC-antizyme activity in mid-S phase, i.e. when the ODC activity was markedly reduced. This finding indicates a role for ODC-antizyme as a regulator of ODC activity during the cell cycle. It has been proposed that ODC-antizyme makes ODC available to a fast-acting proteolytic system (Heller et al., 1976; Seely and Pegg, 1983). Considering the antiproliferative effects of synthetic ODC inhibitors (Heby and Jänne, 1981), modulation of ODC activity by antizyme and other endogenous negative effectors may be important events in the normal regulation of cell growth and division.

LITERATURE CITED

- Anehus, S., Linden, M., Borg, Å., Långström, E. and Heby, O. (1983) Cell cycle dependent induction of ornithine decarboxylase and its antizyme in Ehrlich ascites tumor cells separated by centrifugal elutriation. In: *Advances in Polyamine Research*. U. Bachrach, A. Kaye and R. Chayen eds. Raven Press, New York, Vol. 4, pp. 707-712.
- Anehus, S., Pohjanpelto, P., Baldetorp, B., Långström, E. and Heby, O. (1984) Polyamine Starvation Prolongs the S and G2 Phases of Polyamine-Dependent (Arginase-Deficient) CHO Cells. *Mol. Cell. Biol.*, 4:915-922.
- Atmar, V.J. and Kuehn, G.D. (1981) Phosphorylation of ornithine decarboxylase by a polyamine-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.*, 78:5518-5522.
- Canellakis, E.S., Kyriakidis, D.A., Heller, J.S. and Pawlak, J.W. (1981) The complexity of regulation of ornithine decarboxylase. *Med. Biol.*, 59:279-285.
- Canellakis, E.S., Viceps-Madore, D., Kyriakidis, D.A. and Heller, J.S. (1979) The regulation and function of ornithine decarboxylase and of the polyamines. In: *Current Topics in Cellular Regulation*. B.L.Horecker and E.R. Stadtman eds. Academic Press, New York, Vol. 15, pp. 155-202.
- Clark, J.L. and Fuller, J.L. (1976) Pyridoxal 5'-phosphate and the regulation of ornithine decarboxylase activity and stability. *Eur. J. Biochem.*, 67:303-314.

- Dean, P.N. (1980) A simplified method of DNA distribution analysis. *Cell Tissue Kinet.*, 13:299-308.
- Delcros, J.-G., Roch, A.-M. and Quasch, G. (1984) The competitive inhibition of tissue transglutaminase by α -difluoromethylornithine. *FEBS Lett.*, 171:221-226.
- Folk, J.E. (1980) Transglutaminases. *Annu. Rev. Biochem.*, 49, 517-531.
- Fujita, K., Murakami, Y. and Hayashi, S. (1982) A macromolecular inhibitor of the antizyme to ornithine decarboxylase. *Biochem. J.*, 204:647-652.
- Grabske, R.J., Lake, S., Gledhill, B.L. and Meistrich, M.L. (1975) Centrifugal elutriation: Separation of spermatogenic cells on the basis of sedimentation velocity. *J. Cell. Physiol.*, 86:177-190.
- Heby, O., Gray, J.W., Lindl, P.A., Marton, L.J. and Wilson, C.B. (1976) Changes in L-ornithine decarboxylase activity during the cell cycle. *Biochem. Biophys. Res. Commun.*, 71: 99-105.
- Heby, O. and Jänne, J. (1981) Polyamine antimetabolites: Biochemistry, specificity, and biological effects of inhibitors of polyamine synthesis. In: *Polyamines in Biology and Medicine*. D.R. Morris and L.J. Marton, eds. Marcel Dekker, Inc., New York, pp. 243-310.
- Heller, J.S., Fong, W.F. and Canellakis, E.S. (1976) Induction of a protein inhibitor to ornithine decarboxylase by the end products of

its reaction. Proc. Natl. Acad. Sci. U.S.A., 73:1858-1862.

Hogan, B.L.M., McIlhinney, A. and Murden, S. (1974) Effect of growth conditions on the activity of ornithine decarboxylase in cultured hepatoma cells. II. Effect of serum and insulin. J. Cell. Physiol., 83:353-358.

Jänne, J., Pösö, H. and Raina, A. (1978) Polyamines in rapid growth and cancer. Biochim. Biophys. Acta, 473:241-293.

Jänne, J. and Williams-Ashman, H.G. (1971) On the purification of L-ornithine decarboxylase from rat prostate and effects of thiol compounds on the enzyme. J. Biol. Chem., 246:1725-1732.

Kaye, A.M. and Yariv, M. (1983) Ornithine decarboxylase and ornithine decarboxylase inactivating factor in rat organs. In: Advances in Polyamine Research. U. Bachrach, A. Kaye and R. Chayen eds. Raven Press, New York, Vol. 4, pp. 693-703.

Kitani, T. and Fujisawa, H. (1984) Purification and some properties of a protein inhibitor (antizyme) of ornithine decarboxylase from rat liver. J. Biol. Chem. 259:10036-10040.

Linden, M., Andersson, G. and Heby, O. (1980) Putrescine administration reduces the rate of DNA synthesis in Ehrlich ascites tumor cells in vivo. Int. J. Biochem., 12:387-393.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem., 193:265-275.

McCann, P.P. (1980) Regulation of ornithine decarboxylase in eukaryotes. In: Polyamines in Biomedical Research. J.M. Gaugas, ed. Wiley and Sons Ltd., New York, pp. 109-123.

McCann, P.P., Tardif, C. and Mamont, P.S. (1977) Regulation of ornithine decarboxylase by ODC-antizyme in HTC cells. Biochem. Biophys. Res. Commun., 75:948-954.

Mitchell, J.L.A. (1981) Post-translational controls of ornithine decarboxylase activity. In: Advances in Polyamine Research. C.M. Caldarera, V. Zappia and U. Bachrach eds. Raven Press, New York, Vol. 3, pp. 15-26.

Pegg, A.E., Conover, C. and Wrona, A. (1978) Effects of aliphatic diamines on rat liver ornithine decarboxylase activity. Biochem. J., 170:651-660.

Pegg, A.E. and Williams-Ashman, H.G. (1981) Biosynthesis of putrescine. In: Polyamines in Biology and Medicine. D.R. Morris and L.J. Marton, eds. Marcel Dekker, Inc., New York, pp. 3-42.

Russell, D.H. (1980) Ornithine Decarboxylase as a biological and pharmacological tool. Pharmacology, 20:117-129.

Russell, D.H. (1981) Posttranslational modification of ornithine decarboxylase by its product putrescine. Biochem. Biophys. Res. Commun., 99:1167-1172.

Seely, J.E. and Pegg, A.E. (1983) Effect of 1,3-diaminopropane on ornithine decarboxylase enzyme protein in thioacetamidetreated rat liver. *Biochem. J.*, 216:701-707.

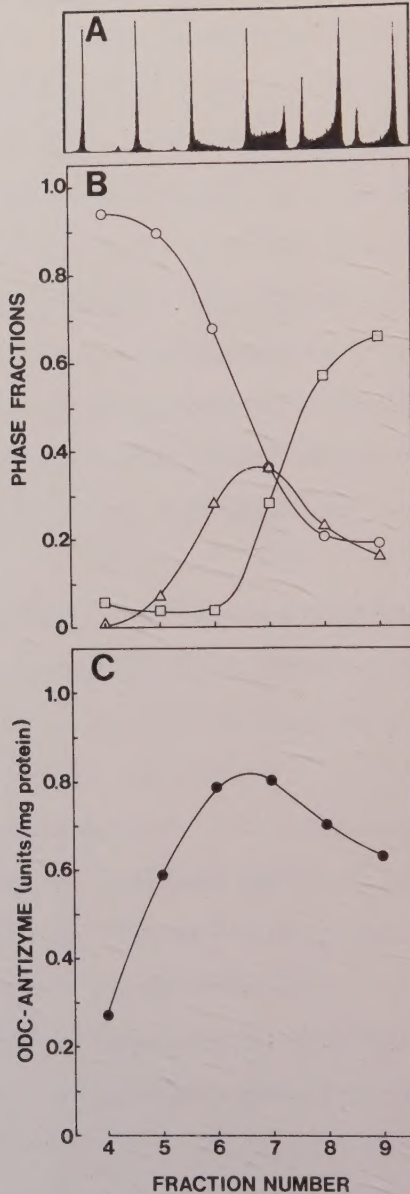


Fig. 2. Changes in ODC-antizyme activity during the Ehrlich ascites tumor cell cycle. Tumor cells, treated with putrescine (25 μ moles/hr per mouse for 4 hr) to induce the release and/or synthesis of ODC-antizyme, were separated according to their size by centrifugal elutriation. Cell fractions (4-9) were analyzed for their cell cycle distribution (A, B) and their ODC-antizyme activity (C). The distribution of cells among the G1 (○), S (Δ) and G2 (□) phases of the cell cycle (B) was calculated from DNA histograms (A) obtained by flow cytometry.

